# GENETIC VACCINE AGAINST HUMAN IMMUNODEFICIENCY VIRUS
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## BACKGROUND OF THE INVENTION

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### Relationship to Parent and Copending Applications

This application is a continuation-in-part of PCT application entitled "GENETIC VACCINE THAT MIMICS NATURAL VIRAL INFECTION AND INDUCES LONG-LASTING IMMUNITY TO PATHOGEN", Application Serial

10 No.: PCT US01/18238, Filed: June 4, 2001, which is a continuation-in-part of U.S. patent application entitled "GENETIC VACCINE THAT MIMICS NATURAL VIRAL INFECTION AND INDUCES LONG-LASTING IMMUNITY TO PATHOGEN", Application Serial No.: 09/585,599, Filed: June 2, 2000. The above applications are incorporated herein by reference.

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### Field of the Invention

This invention relates to vaccines for stimulating immune responses in human and other hosts, and, in particular, relates to recombinant viruses that express heterologous antigens of human immunodeficiency virus (HIV) in a

20 host and elicit immune response to HIV infection.

### Background of the Invention

Current techniques for developing vaccines are largely based on the concept of using denatured virus or purified viral proteins made from bacteria.

25 These types of vaccines may be effective for only a limited number of infectious agents, and the protection rates are limited.

For viruses that contain membrane (envelope) glycoproteins (GPs), including the Ebola virus and the HIV virus, use of denatured virus or purified viral proteins often does not work satisfactorily. There may be several

30 reasons for this. First, the GPs of these viruses are sensitive to the denaturing procedures so that the epitopes of the proteins are altered by the denaturing process. Second, the sugar moieties of the GPs are important antigenic determinants for neutralizing antibodies. In comparison, proteins

made in bacteria are not properly glycosylated and can fold into somewhat different structures that can have antigenecities different from those of the natural viral proteins. Further, many vaccines that are based on attenuated or denatured virus provide a weak immune response to poorly immunogenic

5  antigens. In addition, the vaccine preparations frequently offer only limited protection, not life-long immunity as desired.

Other vaccine approaches express antigens by plasmids directly injected into the body, the so-called naked DNA or DNA vaccine technology. These methods involve the deliberate introduction of a DNA plasmid carrying

10  an antigen-coding gene by transfecting cells with the plasmid in vivo. The plasmid expresses the antigen that causes an immune response. The immune response stimulated by DNA vaccine can be very inefficient, presumably due to low levels of uptake of the plasmid and low levels of antigen expression in the cells. DNA vaccines are also characterized by an

15  extremely short antigen expression period due to vector degradation. In addition, DNA vaccines are difficult and costly to produce in large amounts.

Replication-competent, live vaccinia viruses have also been modified for expression of the genes for hepatitis B (HBV), human immunodeficiency virus (HIV), influenza and malaria antigens. In some instances, though, the

20  immune response of recombinant vaccines is often of limited nature and magnitude. Thus, for example, while peripheral immunization with vaccinia influenza recombinants provides good protection against lower respiratory tract infections, it fails to induce immunity in the upper respiratory tract. On the other hand, peripheral immunization with recombinant vaccines may

25  prove ineffective when local rather than systemic immunity is required, as in, for example, the gastro-intestinal tract.

Vaccination with recombinant vaccinia virus expressing Ebola virus GP has been attempted to confer partial protection in guinea pigs. Gilligan, K.J., et al., *Vaccines*, 97:87-92 (1997). Vaccination with DNA constructs

30  expressing either GP or nucleocapsid protein (NP) protects mice from lethal challenge with Ebola virus. Vanderzanden, L., et al., *Virology*, 246(1):134-44 (1998). However, each of these approaches has its own set of limitations that make them less then ideal choices for Ebola virus vaccines in humans. For

example, vaccinia virus rapidly kills vector-infected cells. Consequently, the vaccine antigen is expressed for only a short time. However, the major limitation for this type of approaches is that the replication of vaccina virus causes the immune system to react mainly to the vaccinia proteins, only small

5    portion of the immune responses is targeted to the antigen of the pathogenic virus. This phenomenon has been termed "antigen dilution".

Previous attempts to remedy these deficiencies, including expression of vaccine antigens through viruses having stronger promoters, such as poxvirus, have not met with significant success.

10    As yet, no vaccine has been effective in conferring protection against HIV infection. Attempts to develop vaccines have thus far failed. Certain antibodies reactive with HIV, notably anti-GP160/120 are present at high levels throughout both the asymptomatic and symptomatic phases of the HIV infection, suggesting that rather than playing a protective role, such

15    antibodies may in fact promote the attachment and penetration of the virus into the host cell. More significantly, current vaccines do not induce efficient cellular responses against the infected cells, the source of newly released virions.

## SUMMARY OF THE INVENTION

Genetic viral vaccines are provided. These vaccines are designed to mimic natural infection of pathogenic viruses without causing diseases that are naturally associated with the pathogenic viruses in a host to be immunized, such as human, domestic animals and other mammals.

The vaccines are recombinant benign viruses that are replication deficient or incompetent. The benign viruses may be designed to express antigens from a wide variety of pathogens such as viruses, bacteria and parasites, and thus may be used to treat this wide variety of viruses, bacteria, and parasites that natively express these antigens. Infection of the benign virus causes host cells to express the antigens of the pathogenic virus and presents the antigen in its natural conformation and pathway as if the cell were infected by the pathogenic virus, and induces a strong and long-lasting immune response in the host.

In one embodiment, a recombinant benign virus is provided for eliciting an immune response in a host infected by the virus. The recombinant virus comprises: an antigen sequence heterologous to the benign virus that encodes a viral antigen from a pathogenic virus, expression of the viral antigen eliciting an immune response directed against the pathogenic virus and cells expressing the viral antigen in the host upon infection of the host by the recombinant virus; and an immuno-stimulator sequence heterologous to the benign virus that encodes an immuno-stimulator whose expression in the host enhances the immunogenicity of the viral antigen. The recombinant virus is replication-incompetent and does not cause disease that is associated with the pathogenic virus in the host

In a variation of the this embodiment, the recombinant benign virus may be a replication-incompetent virus such as adenovirus, adeno-associated virus, SV40 virus, retrovirus, herpes simplex virus or vaccinia virus. Preferably, the benign virus does not have the pathologic regions of the native progenitor of the benign virus but retains its infectivity.

In a preferred embodiment, the benign virus is a replication-incompetent adenovirus, more preferably adenovirus type 5. The

heterologous antigen sequence may be positioned in the E1, E3 or E4 region of the adenovirus. The immuno-stimulator sequence may be positioned in the E4, E3 or E1 region of the adenovirus.

5  In a variation of the preferred embodiment, the heterologous antigen sequence and the immuno-stimulator sequence are positioned in the E1, E3 or E4 region of the adenovirus, where the heterologous antigen sequence and the immuno-stimulator sequence are expressed from a promoter bicistronically via an internal ribosomal entry site or via a splicing donor-acceptor mechanism.

10  Expression of the viral antigen or the immuno-stimulator may be controlled by a promoter homologous to the native progenitor of the recombinant virus. Alternatively, expression of the viral antigen may be controlled by a promoter heterologous to the native progenitor of the recombinant virus. For example, the promoter heterologous to the native progenitor of the recombinant virus may be a eukaryotic promoter such as insulin promoter, human cytomegalovirus (CMV) promoter and its early promoter, simian virus SV40 promoter, Rous sarcoma virus LTR promoter/enhancer, the chicken cytoplasmic β-actin promoter, and inducible promoters such as the tetracycline-inducible promoter.

20  The pathogenic virus may be any pathogenic virus that causes pathogenic effects or disease in human or other animals. Thus, the recombinant benign virus can be used as a vaccine for protecting the host from infection of the pathogenic virus.

In a variation, the pathogenic virus may be various strains of human immunodeficiency virus (HIV), such as HIV-1 and HIV-2. The viral antigen may be an HIV glycoprotein (or surface antigen) such as HIV GP120 and GP41, or a capsid protein (or structural protein) such as HIV P24 protein.

In another variation, the pathogenic virus may be Ebola virus. The viral antigen may be an Ebola glycoprotein or surface antigen such as Ebola GP1 or GP2 protein.

In yet another variation, the pathogenic virus may be hepatitis virus such as hepatitis A, B, C, D or E virus. For example, the viral antigen may be a surface antigen or core protein of hepatitis B virus such as the small

hepatitis B surface antigen (SHBsAg) (also referred to as the Australia antigen), the middle hepatitis B surface antigen (MHBsAg) and the large hepatitis B surface antigen (LHBsAg). The viral antigen may be a surface antigen or core protein of hepatitis C virus such as NS3, NS4 and NS5

5 antigens.

In yet another variation, the pathogenic virus may be a respiratory syncytial virus (RSV). For example, the RSV viral antigen may be the glycoprotein (G-protein) or the fusion protein (F-protein) of RSV, for which the sequences are available from GenBank.

10 In yet another variation, the pathogenic virus may be a herpes simplex virus (HSV) such as HSV-1 and HSV-2. For example, the HSV viral antigen may be the glycoprotein D from HSV-2.

In yet another variation, the viral antigen may be a tumor antigen, such as Her 2 of breast cancer cells and CD20 on lymphoma cells, a viral

15 oncogene such as E6 and E7 of human papilloma virus, or a cellular oncogene such as mutated ras.

It is noted that, other virus-associated proteins or antigens are readily available to those of skill in the art. Selection of the pathogenic virus and the viral antigen associated with the pathogenic virus is not a limiting factor in this

20 invention.

The recombinant virus also expresses an immuno-stimulator to mimic cytokine-releasing response of a host cell upon viral infection and further augments the immune response to the viral antigen co-expressed from the recombinant virus. The immuno-stimulator may preferably be a cytokine.

25 Examples of cytokine include, but are not limited to, interleukin-2, interleukin-8, interleukin-12, β- interferon, λ-interferon, γ-interferon, granulocyte colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF).

The viral antigen may be a full-length antigenic viral protein or a portion

30 of the antigenic viral protein that contains the predominant antigen, neutralizing antigen, or epitope of the pathogenic virus. Alternatively, the viral antigen contains the constant region of glycoproteins of at least two strains of the pathogenic virus.

In a variation, the viral antigen may be a modified antigen that is mutated from a glycoprotein of the pathogenic virus such that the viral antigen is rendered non-functional as a viral component but retains its antigenicity. Such modification of the viral antigen includes deletions in the proteolytic

5    cleavage site of the glycoprotein, and duplications and rearrangement of immunosuppressive peptide regions of the glycoprotein.

In another embodiment, a recombinant adenovirus is provided for eliciting an immune response in a host infected by the virus. The recombinant virus comprises: an antigen sequence heterologous to

10   adenovirus and encoding a viral antigen from a pathogenic virus, expression of the viral antigen eliciting an immune response directed against the viral antigen upon infection of the host by the recombinant adenovirus.

In a preferred variation of the embodiment, the recombinant virus is a replication-incompetent adenovirus. In particular, the pathogenic virus is HIV,

15   including various types (e.g., HIV-1 and HIV-2), strains (e.g, strain BH10 and pNL4-3 of HIV-1), isolates, clades within a group of isolates (e.g., clade A, B, C, D, E, F, and G of group M of HIV-1 isolates) of HIV. The viral antigen may be a 1) HIV glycoprotein (or surface antigen) such as HIV envelope protein Env, either full length wild type (gp160), truncated (e.g, gp120 and gp41), or

20   modified with insertions, deletions or substitutions; 2) HIV structural protein Gag, either full length wild type, modified, or protease-processed products or fragments in various forms (e.g., natural, secreted, or membrane bound forms of HIV capsid proteins such as HIV p24 and p17; and 3) HIV regulatory proteins such as Tat, Vif, Nef, and Rev.

25   According to this variation, the HIV antigen is an HIV envelop protein encoded by a polynucleotide selected from the group consisting of SEQ ID NOs: 14, 16, 20, 21, 22, 23, and 24. The polynucleotide may further encode HIV regulatory proteins such as Tat, Vif, Nef, and Rev.

Also according to the variation, the HIV antigen is a modified HIV

30   envelope protein that includes multiclade variable loops. Preferably, the multiclade variable loops are V3 loops from various clades such as clade A, B, C, D, E, F, and G of group M of HIV-1 isolates. The modified HIV envelope protein that includes multiclade variable loops may include two or

more V3 loops from different HIV clades, preferably V3 loops encoded by polynucleotides selected from the group consisting of SEQ ID NOs: 25, 26, 27, 28, 29, 30, and 31. More preferably, the modified HIV envelope protein that includes multiclade variable loops is encoded by a polynucleotide

5  selected from the group consisting of SEQ ID NOs: 32, 52, and 54.

Also according to the variation, the HIV antigen is an HIV structural protein. The HIV structural protein may be a full length wild type Gag encoded by SEQ ID NO: 17, or a proteolytic fragment of Gag such as p17/24, p17 and p24. The fragment p17/24 may be in natural form and encoded by

10  SEQ ID NO: 34, in secreted form and encoded by SEQ ID NO: 34, or in membrane bound form and encoded by SEQ ID NO: 36. The fragment p17 may be in natural form and encoded by SEQ ID NO: 40, in secreted form and encoded by SEQ ID NO: 41, or in membrane bound form and encoded by SEQ ID NO: 42. Similarly, p24 may be in natural form and encoded by SEQ

15  ID NO: 46, in secreted form and encoded by SEQ ID NO: 47, or in membrane bound form and encoded by SEQ ID NO: 48.

The recombinant virus may further comprise a polynucleotide encoding an HIV protease PI such as SEQ ID NO: 56, expression of which facilitates proteolytic processing of Gag expressed from the same recombinant virus or

20  from another vector. PI may be expressed as a fusion protein with Gag, or separately from a different promoter or from the same promoter for Gag via an IRES or splicing donor/acceptor mechanism.

Optionally, the recombinant virus may further comprise an immuno-stimulator sequence heterologous to the recombinant virus that encodes an

25  immuno-stimulator whose expression in the host enhances the immunogenicity of the viral antigen.

The present invention also provides viral vaccines that present multiple antigens to the host to further mimic natural infection of a native pathogenic virus and induce strong and long-lasting immune response to various strains

30  or types of the pathogenic virus in the host.

In one embodiment, a recombinant virus is provided as a viral vaccine for eliciting an immune response in a host infected by the virus. The recombinant virus comprises: a plurality of antigen sequences heterologous

to the recombinant virus, each encoding a viral antigen from a same pathogenic virus, different strains of a pathogenic virus, or different kinds of pathogenic viruses, expression of the plurality of the antigen sequences eliciting an immune response directed against the viral antigen and cells

5     expressing the viral antigen in the host upon infection of the host by the recombinant virus. The recombinant virus may preferably be replication-incompetent and not cause malignancy in the host naturally associated with pathogenic virus.

According to the embodiment, the recombinant virus may be any virus,

10    preferably replication-incompetent adenovirus, adeno-associated virus, SV40 virus, retrovirus, herpes simplex virus or vaccinia virus. The benign virus may also preferably have the pathologic regions of the native progenitor of the benign virus deleted but retain its infectivity.

Also according to the embodiment, the plurality of the antigen

15    sequences may be multiple copies of the same antigen sequence or multiple antigen sequences that differ from each another.

In a variation of the embodiment, at least two of the plurality of the antigen sequences are expressed from a promoter bicistronically via an internal ribosomal entry site or via a splicing donor-acceptor mechanism.

20    Optionally, at least two of the plurality of the antigen sequences are expressed from a promoter to produce a fusion protein.

Also according to the embodiment, the viral genome further comprises at least one promoter heterologous to the native progenitor of the recombinant virus that controls the expression of at least two of the plurality of

25    the antigen sequences. Examples of the promoter heterologous to the native progenitor of the recombinant virus include, but are not limited to, insulin promoter, CMV promoter and its early promoter, SV40 promoter, retrovirus LTR promoter/enhancer, the chicken cytoplasmic β-actin promoter, and inducible promoters such as tetracycline-inducible promoter.

30    Also according to the embodiment, the plurality of antigen sequences may be a combination of antigens from at least two strains of the pathogenic virus.

Optionally, the plurality of antigen sequences may be a combination of antigens from at least two different pathogenic viruses. For example, the plurality of antigen sequences may be a combination of antigens from HIV-1, HIV-2, herpes simplex virus type 1, herpes simplex virus type 2, Ebola virus,

5  Marburg virus, and hepatitis A, B, C, D, and E viruses.

In a variation of the embodiment, the recombinant virus may further comprise one or more immuno-stimulator sequences that are heterologous to the benign virus and encodes an immuno-stimulator whose expression in the host enhances the immunogenicity of the viral antigen. For example, the

10  immuno-stimulator may be a cytokine. Examples of the cytokine include, but are not limited to, interleukin-2, interleukin-4, interleukin-12, $\beta$-interferon, $\lambda$-interferon, $\gamma$-interferon, G-CSF, and GM-CSF.

According to the variation, the one or more immuno-stimulator sequences may be multiple copies of the same immuno-stimulator sequence

15  or multiple immuno-stimulator sequences that differ from each other.

Optionally, at least two of the immuno-stimulator sequences may be expressed from a promoter multicistronically via an internal ribosomal entry site or via a splicing donor-acceptor mechanism. Alternatively, at least two of the immuno-stimulator sequences may be expressed from a promoter to form

20  a fusion protein.

The present invention also provides genetic vaccines that elicit strong and long-lasting immune response to pathogenic bacteria. In one embodiment, a recombinant virus is provided as a genetic bacteria vaccine for eliciting an immune response in a host infected by the recombinant virus.

25  The recombinant virus comprises: a plurality of antigen sequences heterologous to the recombinant virus, each encoding a bacterial antigen from a pathogenic bacteria, expression of the plurality of the bacterial antigen sequences eliciting an immune response directed against the bacterial antigen and cells expressing the bacterial antigen in the host upon infection of

30  the host by the recombinant virus. The recombinant virus may preferably be replication-incompetent and not cause malignancy naturally associated with the pathogenic bacteria in the host.

The pathogenic bacteria may be any pathogenic bacteria that causes pathogenic effects or diseases in a host, such as *bacillus tuberculoses*, *bacillus anthracis*, and *spirochete Borrelia burgdorferi* that causes the Lyme disease in animals. The plurality of antigen sequences may encode lethal

5    factors, protective antigen, edema factors of the pathogenic bacteria, or combinations thereof.

The present invention also provides vaccines against parasites that elicit strong and long-lasting immune response to pathogenic parasites. In one embodiment, a recombinant virus is provided as a parasite vaccine for

10    eliciting an immune response in a host infected by the recombinant virus. The recombinant virus comprises: a plurality of antigen sequences heterologous to the benign virus, each encoding a parasitic antigen from a pathogenic parasite, expression of the plurality of the parasitic antigen sequences eliciting an immune response directed against the parasitic

15    antigen and cells expressing the parasitic antigen in the host upon infection of the host by the recombinant virus. The recombinant virus may preferably be replication-incompetent and not cause a malignancy naturally associated with the pathogenic parasite in the host.

The pathogenic parasite may be any pathogenic parasites that cause

20    pathogenic effects or diseases in a host, such as malaria and protozoa such as Cryptosporidium, Eimeria, Histomonas, Leucocytozoon, Plasmodium, Toxoplasma, Trichomonas, Leishmania, Trypanosoma, Giardia, Babesia, and Theileria. The plurality of antigen sequences may encode coat proteins, attachment proteins of the pathogenic parasites, or combinations thereof.

25    The present invention also provides pharmaceutical compositions that include the viral vaccines of the present invention. The pharmaceutical composition may include any of the recombinant viruses described above and a pharmaceutically acceptable carrier or diluent.

The pharmaceutical composition may also include an adjuvant for

30    augmenting the immune response to the viral antigen expressed from the recombinant virus. Examples of the adjuvant include, but are not limited to, bacillus Calmette-Guerin, endotoxin lipopolysaccharide, keyhole limpet hemocyanin, interleukin-2, GM-CSF, and cytoxan.

The present invention also relates to kits. These kits may include any one or more vaccines according to the present invention in combination with a composition for delivering the vaccine to a host and/or a device, such as a syringe, for delivering the vaccine to a host.

5    The present invention also provides methods for enhancing the immunity of a host with the recombinant viruses described above.

In one embodiment, the method comprises: administering to the host a recombinant virus in an amount effective to induce an immune response. The in the recombinant virus comprises: an antigen sequence heterologous to 

10   the recombinant virus that encodes a viral antigen from a pathogenic virus, expression of the viral antigen eliciting an immune response directed against the viral antigen and cells expressing the viral antigen in the host upon infection of the host by the recombinant virus; and an immuno-stimulator sequence heterologous to the benign virus that encodes an immuno-

15   stimulator whose expression in the host enhances the immunogenicity of the viral antigen. The recombinant virus may preferably be replication-incompetent and not cause malignancy naturally associated with the pathogenic virus in the host.

The recombinant virus may be administered to the host via any 

20   pharmaceutically acceptable route of administration. The recombinant virus may be administered to the host via a route of intramuscular, intratracheal, subcutaneous, intranasal, intradermal, rectal, oral and parental administration.

In another embodiment, a method is provided for enhancing the 

25   immunity of a host to a pathogenic virus with multiple antigens. The method comprises: administering to the host a recombinant virus in an amount effective to induce an immune response. The recombinant virus comprises: a plurality of antigen sequences heterologousto the benign virus, each encoding a viral antigen from a pathogenic virus, expression of the plurality of 

30   the antigen sequences eliciting an immune response directed against the viral antigen and cells expressing the viral antigen in the host upon infection of the host by the recombinant virus. The recombinant virus may preferably be

replication-incompetent and not cause malignancy naturally associated with the pathogenic virus in the host.

Optionally, the recombinant virus may further comprise one or more immuno-stimulator sequences heterologous to the recombinant virus that

5    encodes an immuno-stimulator whose expression in the host enhances the immunogenicity of the viral antigen.

In yet another embodiment, a method is provided for enhancing the immunity of a host to a pathogenic virus by using multiple recombinant viral vaccines (or viruses). Multiple recombinant viruses may carry different

10   antigens in each recombinant virus. The multiple recombinant viruses may be administered simultaneously or step-wise to the host.

The method comprises: administering to a host a first and second recombinant viruses in an amount effective to induce an immune response. The first recombinant virus comprises: an antigen sequence heterologous to

15   the first recombinant virus that encodes a viral antigen from a pathogenic virus, expression of the viral antigen eliciting an immune response directed against the viral antigen and cells expressing the viral antigen in the host upon infection of the host by the recombinant virus. The second recombinant virus comprises: an immuno-stimulator sequence heterologous to the

20   recombinant virus that encodes an immuno-stimulator whose expression in the host enhances the immunogenicity of the viral antigen. The first and second recombinant viruses may preferably be replication-incompetent and not cause a malignancy naturally associated with the pathogenic virus in the host.

25   According to the embodiment, the first and second recombinant virus may be any benign virus, such as replication-incompetent adenovirus, adeno-associated virus, SV40 virus, retrovirus, herpes simplex virus and vaccinia virus. Optionally, both the first and second recombinant viruses may be replication-incompetent adenovirus. Also optionally, one of the first and

30   second recombinant viruses may be recombinant adenovirus and the other may be recombinant vaccinia virus.

In yet another embodiment, a method is provided for enhancing the immunity of a host to a pathogen. The method comprises: administering to

the host a recombinant virus and one or more immuno-stimulators. The recombinant virus may be any of the recombinant viruses described above. In particular, the recombinant virus comprises one or more antigen sequences heterologous to the recombinant virus that encode one or more

5    antigens from the pathogen. Expression of the antigen elicits an immune response directed against the antigen and cells expressing the antigen in the host upon infection of the host by the recombinant virus. The recombinant virus is preferably replication-incompetent and does not cause a malignancy naturally associated with the pathogen in the host. The pathogen may be a

10   pathogenic virus such as HIV, hepatitis virus and Ebola virus, a pathogenic bacteria or parasite.

According to this embodiment, the immuno-stimulator may be any molecule that enhances the immunogenicity of the antigen expressed by the cell infected by the recombinant virus. Preferably, the immuno-stimulator is a

15   cytokine, including, but not limited to interleukin-2, interleukin-8, interleukin-12, β- interferon, λ-interferon, γ-interferon, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, and combinations thereof. The cytokine may be administered into the host in a form of purified protein alone or formulated with one or more pharmaceutically acceptable

20   excipients. Alternatively, the cytokine may be administered in a form of expression vector that expresses the coding sequence of the cytokine upon transfecting or transducing the cells of the host.

According to any of the above embodiments of the methods, the method may further comprising: administering to the host the recombinant

25   virus again to boost the immune response. Such a booster inoculation with the recombinant virus is preferably conducted several weeks to several months after the primary inoculation. To insure sustained high levels of protection against infection or an efficacious treatment of the disease(s) caused by infection of the pathogen, it may be helpful to readminister the

30   booster immunization to the host at regular intervals, for example, once every several years. The recombinant virus administered in the booster immunization may be the same as or different from the recombinant virus administered in the primary immunization.

It should be noted that modifications and changes can be made in the DNA sequence of any of the above-described antigens and immuno-stimulators included in the recombinant virus and still maintain functional equivalence of the mutant. The resulting mutants fall within the scope of the present invention.

# BRIEF DESCRIPTION OF THE FIGURES

**Figures 1A-1C** illustrate an example of how to construct a genetic vaccine of the present invention.

5      **Figure 1A** illustrates an example of a shuttle vector pLAd.Antigen carrying multiple antigen genes such as Antigen 1 and Antigen 2 which can be expressed from a $CMV_{ie}$ promoter bicistronically via a splicing donor-acceptor mechanism at the SD and SA sites.

     **Figure 1B** illustrates an example of a shuttle vector pRAd.Cytokines

10    carrying multiple cytokine genes such as IL-2, INF, and IL-8 genes which can be expressed from a $CMV_{ie}$ promoter bicistronically via an internal ribosomal entry site IRES and a splicing donor-acceptor mechanism at the SD and SA sites.

     **Figure 1C** illustrates an example of constructing a genetic vaccine by

15    ligating with an adenoviral backbon with a fragment that is derived from the shuttle vector pLAd.Antigen and contains multiple antigen genes and a fragment that is derived from the shuttle vector pRAd.Cytokines and contains multiple cytokine genes.

     **Figure 2** illustrates the wild-type GP gene, which encodes the two

20    forms of glycoproteins (sGP and GP), contains a RNA editing signal that results in un-edited and edited mRNAs. The sGP is synthesized from an un-edited mRNA and the GP is synthesized from an edited mRNA (having an insertion in one of the seven uridines). **Figure 2** also depicts the modifications made to the RNA to prevent the synthesis of sGP. The RNA

25    editing site is modified from UUU UUU U to UUC UUC UU. This modification removes the editing signal and results in the mRNA coding only for the GP.

     **Figure 3** illustrates the modification of the immunosuppressive peptide (IS) located in GP2. **Figure 3A** shows the wild type GP. **Figure 3B** shows GP with the 10 amino acid deletion of the IS peptide. **Figure 3C** shows the IS

30    peptide, which is split, reversed and duplicated. Abbreviations: FP, Fusion peptide; IS, Immunosuppressive peptide; TM, Transmembrane domain.

Figures **4A** and **4B** illustrate a procedure used to create a recombinant adenoviral vector as a genetic vaccine against Ebola virus.

**Figures 4A** illustrates a shuttle vector pLAd/EBO-GP carrying the GP gene of Ebola virus an antigen, and a shuttle vector pRAdIL2,4 carrying the

5    IL-2 and IL-4 gene.

**Figure 4B** illustrates the construction of a recombinant adenoviral vector by ligating an adenoviral backbone with a fragment that is derived from the shuttle vector pLAd/EBO-GP and contains the GP gene and a fragment that is derived from the shuttle vector pRAdIL2,4 and contains IL-2 and IL-4

10   genes.

**Figure 5** illustrates a complex adenoviral vector as an example of the genetic vaccine of the present invention. The Ebola viral GP gene is expressed by a CMVie promoter in the E1 region. The GP gene is followed by INF-$\gamma$ and GM-CSF which are expressed by two IRES sequences. This

15   configuration allows for the expression of three proteins from a single mRNA. Expression of IL-2 and IL-4 is controlled by a second CMVie promoter as a bi-cistronic cassette, and followed by a second bi-cistronic cassette that expressed the two subunits of IL12 in the E4 region by a SV40 early promoter.

20   **Figure 6** shows relative titers of antibody against HIV antigens in a group of mice.

**Figure 7** shows relative titers of antibody against HIV antigens in another group of mice.

**Figure 8A-C** show INF-$\gamma$ secretion from activated splenocytes

25   harvested from mice inoculated with adenoviral vectors in response to target cell stimulation.

**Figure 9** shows granzyme A secretion from activated splenocytes harvested from mice inoculated with adenoviral vectors in response to target cell stimulation.

30   **Figure 10A** shows relative titers of antibody against HBV surface antigen in a group of mice.

**Figure 10B** shows relative titers of antibody against HBV surface antigen in another group of mice.

**Figure 11A** shows relative titers of antibody against HBV core antigen in a group of mice.

5       **Figure 11B** shows relative titers of antibody against HBV core antigen in another group of mice.

**Figure 12A** shows relative titers of antibody against HIV Gag in mice in week 10 post-immunization with Ad-3C/E$^m\Delta$C$\Delta$T$^{300}$-G.

**Figure 12B** shows relative titers of antibody against HIV Gag in mice in

10     week 14 post-immunization/week 3 post-boost with Ad-3C/E$^m\Delta$C$\Delta$T$^{300}$-G.

**Figure 13A** shows relative titers of antibody against HIV Gag in mice in week 10 post-immunization with Ad-3C/E$^m\Delta$C$\Delta$T$^{99}$-G.

**Figure 13B** shows relative titers of antibody against HIV Gag in mice in week 14 post-immunization/week 3 post-boost with Ad-3C/E$^m\Delta$C$\Delta$T$^{99}$-G.

15       **Figure 14A** shows results of the granzyme A assays for serie 1 mice at week 4, 6, 8 post-immunization and week 12/1, 13/2, 14/3 (prime/boost) post-secondary inoculation with Ad.3C.env.gag.

**Figure 14B** shows the results of the granzyme A assays for serie 2 mice at week 2, 4, 6, 8 post-immunization with Ad.3C.env.gag.

20       **Figure 15A** shows the ELISPOT results for the four mice in serie 1 at week 13/2 post-prime/boost with Ad.3C.env.gag.

**Figure 15B** shows the ELISPOT results for the four mice in serie 1 at week 13/2 post-prime/boost with Ad.3C.env.rev.gag.

**Figure 16A** illustrates a shuttle vector pLAd-E.T.R.

25       **Figure 16B** illustrates a shuttle vector pRAd-ORF6-IL2.

**Figure 17A** illustrates a shuttle vector pRAd-ORF6-cmv- E$^m\Delta$C$\Delta$T$^{300}$-G.

**Figure 17B** illustrates a shuttle vector pLAd-3C.

**Figure 18** illustrates a shuttle vector pRAd-E$^m\Delta$C$\Delta$T$^{99}$.T.R-G

**Figure 19A** illustrates a shuttle vector pLAd- E$^m\Delta$V$_{1,2}$ $\Delta$C $\Delta$T.T.R-IL2.

30       **Figure 19B** illustrates a shuttle vector pRAd-ORF6-G.IL2.

**Figure 20** illustrates a shuttle vector pLAd-E$^m\Delta$C.T.R.N.

**Figure 21** illustrates a shuttle vector pLAd-E$^m\Delta$C.N.

**Figure 22** illustrates a shuttle vector pLAd-E$^m$ΔC ΔT$^{300}$.T.

**Figure 23A** illustrates a shuttle vector pLAd-E$^m$ΔC.

**Figure 23B** illustrates a shuttle vector pRAd-ORF6-E$^m$ΔC.

**Figure 24** illustrates a process for constructing a multi-clade insert by

5    PCR.

**Figure 25** illustrates a shuttle vector pLAd-E$^m$.V3.

**Figure 26** illustrates a shuttle vector pLAd-E$^m$.2xV3.

**Figure 27A** illustrates a shuttle vector pRAd-ORF6-p17/p24.

**Figure 27B** illustrates a shuttle vector pRAd-ORF6-p17/p24sec.

10    **Figure 27C** illustrates a shuttle vector pRAd-ORF6-p17/p24MB.

**Figure 28A** illustrates a shuttle vector pRAd-ORF6-p17.

**Figure 28B** illustrates a shuttle vector pRAd-ORF6-p17sec.

**Figure 28C** illustrates a shuttle vector pRAd-ORF6-p17MB.

**Figure 29A** illustrates a shuttle vector pRAd-ORF6-p24.

15    **Figure 29B** illustrates a shuttle vector pRAd-ORF6-p24sec.

**Figure 29C** illustrates a shuttle vector pRAd-ORF6-p24MB.

**Figure 30A-B** illustrate a process of construction of Ad-E$^m$.2xV3$^m$/p17/p24MB.

**Figure 31A-B** illustrate a process of construction of Ad-

20    E$^m$.2xV3$^m$/p17MB.

**Figure 32A-B** illustrate a process of construction of Ad-E$^m$.2xV3$^m$/p24MB.

**Figure 33** illustrates a shuttle vector pLAd-E$^m$ΔCΔT$^{300}$.2xV3$^m$.T.

**Figure 34** illustrates a shuttle vector pLAd-E$^m$ΔCΔT$^{99}$.2xV3$^m$.T.R.

25    **Figure 35** illustrates a shuttle vector pRAd-ORF6-G.PI.

**Figure 36** illustrates a shuttle vector pRAd-ORF6-G-PI.

**Figure 37** illustrates a cloning vector SD/SA1.2.3

**Figure 38** shows DNA sequence encoding Env/Tat/Rev from HIV-1 strain BH10.

30    **Figure 39** shows DNA sequence encoding a mutated IL-2 (IL-2ΔX).

**Figure 40** shows DNA sequence encoding a modified Env (E$^m$ΔCΔT (BH10).

**Figure 41A** shows DNA sequence encoding the full length HIV Gag.

**Figure 41B** shows amino acid sequence of the full length HIV Gag.

**Figure 42** shows DNA sequence encoding Env, and full length Tat and Rev.

5        **Figure 43** shows DNA sequence encoding $E^m\Delta V_{1,2} \Delta C \Delta T.T.R.$

**Figure 44** shows DNA sequence encoding $E^m\Delta C.T.R.N.$

**Figure 45** shows DNA sequence encoding $E^m\Delta C.N.$

**Figure 46** shows DNA sequence encoding $E^m\Delta C\Delta T^{300}.T.$

**Figure 47** shows DNA sequence encoding $E^m/ E^m.$

10        **Figure 48** shows DNA sequences of V3 loops of clade B, A, C, D, E, F, and G.

**Figure 49A** shows DNA sequence encoding a modified Env including multi-clade V3 loops.

**Figure 49B** shows amino acid sequence encoding a modified Env

15 including multi-clade V3 loops.

**Figure 50A** shows DNA sequence encoding p17/p24 in natural form, secreted form, and membrane bound form, respectively.

**Figure 50B** shows amino acid sequence of p17/p24 in natural form, secreted form, and membrane bound form, respectively.

20        **Figure 51A** shows DNA sequence encoding p17 in natural form, secreted form, and membrane bound form, respectively.

**Figure 51B** shows amino acid sequence of p17 in natural form, secreted form, and membrane bound form, respectively.

**Figure 52A** shows DNA sequence encoding p24 in natural form,

25 secreted form, and membrane bound form, respectively.

**Figure 52B** shows amino acid sequence of p24 in natural form, secreted form, and membrane bound form, respectively.

**Figure 53A** shows DNA sequence encoding a modified Env including multi-clade V3 loops, and Tat.

30        **Figure 53B** shows amino acid sequence of a modified Env including multi-clade V3 loops, and Tat.

**Figure 54A** shows DNA sequence encoding a modified Env including multi-clade V3 loops, Tat, and Rev.

**Figure 54B** shows amino acid sequence of a modified Env including multi-clade V3 loops, Tat, and Rev.

5          **Figure 55A** shows DNA sequence encoding an HIV protease PI.

**Figure 55B** shows amino acid sequence of an HIV protease PI.

**Figure 56A** shows DNA sequence encoding HIV Gag-PI.

**Figure 56B** shows amino acid sequence of HIV Gag-PI.

**Figure 57** shows PCR primers for cloning V3 loops from multiple HIV

10    clades.

# DETAILED DESCRIPTION OF THE INVENTION

The present invention provides genetic vaccines, pharmaceutical compositions including the vaccines and methods of immunizing a host

5    against infection of a wide range of pathogenic viruses, bacteria and parasites. The genetic vaccines are recombinant benign viruses that are replication deficient and do not cause malignancy in the host to be immunized. Vaccination using the genetic vaccines of the present invention mimics natural viral infection in that the antigen(s) expressed by the cell

10   infected by the genetic vaccine is presented to the host immune system in its natural conformation and by a "inside-out" mechanism, as compared with the conventional "outside-in" approach of vaccination using denatured protein or virus as a vaccine. The recombinant virus is capable of expressing multiple pathogenic antigens, mimicking natural pathogen infection. In particular,

15   multiple pathogenic antigens such as a combination of an HIV envelop protein Env and structural protein Gag, either wildtype or mutant, can be expressed by the recombinant virus to elicit not only humoral immune response (i.e., production of antibody from B cells, helper T cells, and suppressor T cells), but also cellular response by producing cytotoxic T lymphocytes (CTL)

20   directed specifically to these antigens. Further, the pathogenic antigen that is naturally expressed as an intracellular protein can be modified to be secretable and rendered bound to the cell surface, thus better presenting the antigen to the body's immune system. In addition, the cell infected by the genetic vaccine may also release high levels of cytokine, thereby further

25   mimicking the natural response of the cell under stress induced by viral infection and yet not causing pathogenic effects on the cells. Mistaken by such a "signal of pathogenic viral infection", the host immune system mounts a strong immune defense against the antigen presented by the infected cell. Therefore, in a sense, the genetic vaccine of the present invention behaves

30   like a "sheep in wolf's clothing", presenting the viral antigen to induce a strong immune response and yet not causing the detrimental effects that the pathogens would cause on the host. The recombinant viruses of the present invention can not only be used as a vaccine to prevent infection of the

pathogen but also as a therapeutic agent to treat diseases associated with the infection of the pathogen.

In one embodiment, a recombinant virus is provided for eliciting an immune response in a host infected by the virus. The recombinant virus

5    comprises: an antigen sequence heterologous to the recombinant virus and encoding a viral antigen from a pathogenic virus, expression of the viral antigen eliciting an immune response directed against the viral antigen and cells expressing the viral antigen in the host upon infection of the host by the recombinant virus; and an immuno-stimulator sequence heterologous to the

10   recombinant virus and encoding an immuno-stimulator whose expression in the host enhances the immunogenicity of the viral antigen. The recombinant virus is replication-incompetent and does not cause the malignancy naturally associated with the pathogenic virus in the host.

In another embodiment, a recombinant virus is provided as a viral

15   vaccine for eliciting an immune response against multiple antigens in a host infected by the virus. The recombinant virus comprises: a plurality of antigen sequences heterologous to the benign virus, each encoding a different viral antigen from one or more pathogenic viruses, expression of the plurality of the antigen sequences eliciting an immune response directed against the viral

20   antigens and cells expressing the viral antigen in the host upon infection of the host by the recombinant virus. The recombinant virus may preferably be replication-incompetent and not cause the malignancy that is naturally associated with the pathogenic virus(es) in the host.

The vaccines of the present invention can be used to immunize the

25   host against a wide variety and different strains of pathogenic viruses such as HIV-1, HIV-2, herpes simplex virus type 1, herpessimplex virus type 2, Ebola virus, Ebola virus, and hepatitis A, B, C, D, and E viruses, or pathogenic bacteria such as *bacillus tumerculoses* and *bacillus anthracis*.

The recombinant vaccine of the present invention is a recombinant

30   virus that contains nucleic acid sequences encoding one or more viral antigens in the viral genome. When a host is immunized by the recombinant vaccine, i.e., infected by the recombinant virus, the infection of the virus in a host cell results in expression of the viral antigen which is present on the

surface of the infected cell. Since expression of the viral antigen is driven by a strong promoter, expession can be maintained at a high level. Upon recognizing the large amount viral antigen on the cell surface, the host immune system mounts a strong defense against the viral antigen, thereby

5    achieving long-lasting immunity against the pathogenic virus from which the viral antigen is derived.

Compared with immunization with vaccines that are isolated proteins expressed by bacteria, yeast or insect cells, the viral antigen expressed from the recombinant virus of the present invention better mimics the natural viral

10    antigen in its structure and function. Isolated protein vaccine may not adopt the native conformation of the natural viral antigen and may not be properly glycosylated in the bacteria, yeast or insect cells. When such an isolated protein vaccine is injected into the host, this antigen is presented from the outside of the host cell. This conventional "outside-in" approach often does

15    not generate strong, long-lasting immune response, presumably due to the altered antigenicity of the vaccine and quick clearance of the protein vaccine by the immune scavenging cells.

In contrast, the genetic vaccine of the present invention, i.e., the recombinant virus, presents the viral antigen by an "inside-out" mechanism.

20    The viral antigen is expressed after infection of the recombinant virus in the host cells. This better mimics the natural production and presentation of the viral antigen by the pathogenic virus.

By using a replication incompetent virus that is incapable of spreading beyond initially infected cells, the present invention dramatically reduces the

25    risk of side effects that may potentially be generated by using replication-competent, live virus. For example, vaccines based on live vaccinia virus can replicate in the host cells, which can impose a high level of stress on the host cell and eventually lead to cell death.

Moreover, compared to the approach of using attenuated or inactive

30    virus as a vaccine, the process of making the genetic vaccine of the present invention is much safer. Vaccination of a large population of people or animals demand large amounts of vaccines. For virulent viruses such as Ebola virus and HIV, large-scale production of attenuated or inactive virus

from the live virus can pose a great danger to the environment and people who handle the live virus.

The recombinant virus of the present invention can be used to express multiple antigen sequences simultaneously from the same viral vector. Thus,

5    the recombinant virus may encode multiple antigens from the same strain of pathogenic virus, from different strains of the same pathogenic viruses, or from different antigens from different kind of viruses, bacteria or parasites. This enables the vaccines of the present invention to be utilized to immunize against a broad-spectrum of viruses and other infectious agents. Since these

10   multiple antigen sequences are rearranged in the recombinant viral genome, the risk of potential recombination of these viral sequences to generate a pathogenic virus is virtually eliminated.

The genetic vaccine of the present invention also preferably express large amount of immunuo-stimulator, such as cytokine. In a natural process

15   of viral infection, virus-infected cells display viral antigens on their surface in the context of the MHC-I receptor, while viral particles are digested by the professional antigen-presenting cells which display antigens in association with MHC-II receptors. In response to viral infection, a full range of cytokines and interferons are produced, resulting in a strong humoral and cellular

20   response to the viral antigens. At the same, large numbers of memory cells remain to defeat any new infection. In vaccinations using isolated protein vaccines, the protein is quickly cleared by the immune scavenging cells. During this process, only MHC-II antigen presentation occurs and the cytokine-releasing response is absent or greatly diminished. As a result, little

25   cellular response is generated and few "memory" cells are produced.

In comparison, co-expression of viral antigen and cytokine from the recombinant virus of the present invention effectively mimics the natural response of the host cell to viral infection by presenting the antigen on the surface of the infected and producing large amount of immuno-modulating

30   cytokines. With the high levels of cytokine expressed from the host cells infected by the genetic vaccine, the host immune system would be "tricked" to mount a strong response to vaccine, thereby resulting in a longer-lasting immunity.

Additionally, although vaccination with the genetic vaccine mimics the natural viral infection of a pathogenic virus, the vaccine itself is a benign virus that does not have the detrimental effects of the pathogenic virus. For example, infection of a pathogenic virus such as HIV, influenza virus and

5    Ebola virus has profound immuno-suppressing effects on the host, presumably due to the immuno-suppressing functions of the glycoproteins of the virus. According to the present invention, the viral antigen sequence carried by the genetic vaccine is preferred to have its pathogenic or immuno-suppressing regions deleted. In a sense, the genetic vaccine of the present

10   invention behaves like a "sheep in wolf's clothing", presenting the viral antigen to induce strong immune response and yet not causing detrimental effects on the host.

### 1.    The Genetic Vaccines of the Present Invention

15   The present invention is directed to vaccines that mimic the features of a native pathogenic virus, but without eliciting immuno-suppression and pathogenicity, thus causing the host to mount an effective defense, while not being in any actual danger of infection. The genetic vaccines are replication incompetent or defective viruses into which one or more DNA sequences

20   encoding one or more viral antigens are inserted into the regions of the viral genome non-essential to its infectivity. The recombinant virus expresses the viral antigens and elicits a cell-mediated immune response *in vivo* directed against the antigens and cells expressing the antigens.

In one embodiment, a recombinant virus is provided for eliciting an

25   immune response in a host infected by the virus. The recombinant virus comprises: an antigen sequence heterologous to the recombinant virus that encodes a viral antigen from a pathogenic virus, expression of the viral antigen eliciting an immune response directed against the viral antigen and cells expressing the viralantigen in the host upon infection of the host by the

30   recombinant virus; and an immuno-stimulator sequence heterologous to the recombinant virus that encodes an immuno-stimulator whose expression in the host enhances the immunogenicity of the viral antigen. The recombinant

virus is replication-incompetent and does not cause a malignancy naturally associated with the pathogenic virus in the host.

The recombinant virus may be constructed from any virus as long as the native progenitor is rendered replication incompetent. For example,

5    replication-incompetent adenovirus, adeno-associated virus, SV40 virus, retrovirus, herpes simplex virus or vaccinia virus may be used to generate the recombinant virus by inserting the viral antigen into the region non-essential to the infectivity of the recombinant virus. Therefore, it is preferred that the recombinant virus does not have the pathologic regions of the native

10    progenitor of the benign virus but retains its infectivity to the host.

In a preferred embodiment, the recombinant virus is a replication-incompetent adenovirus.

The recombinant adenovirus of the present invention can direct high levels of antigen expression that provide strong stimulation of the immune

15    system. The antigen expressed by cells infected by adenovirus is processed and displayed in the infected cells in a way that mimics pathogen-infected cells. This phase is believed to be very important in inducing cellular immunity against infected cells, and is completely lacking when conventional vaccination approaches are used. Further, the recombinant adenovirus may

20    infect dendritic cells which are very potent antigen-presenting cells. Further, the recombinant adenovirus may also carry genes encoding immuno-enhancing cytokines to further boost immunity. Moreover, the recombinant adenovirus may naturally infect airway and gut epithelial cells in humans, and therefore the vaccine may be delivered through nasal spray or oral ingestion.

25    In addition, the recombinant adenovirus of the present invention should be safe because it is replication-incompetent.

The heterologous antigen sequence may be positioned in the E1, E3 or E4 region of the adenovirus. The immuno-stimulator sequence may be positioned in the E1, E3 or E4 region of the adenovirus.

30    In a variation of the preferred embodiment, the heterologous antigen sequence and the immuno-stimulator sequence are positioned in the E1, E3 or E4 region of the adenovirus, where the heterologous antigen sequence and the immuno-stimulator sequence are expressed from a promoter

bicistronically via an internal ribosomal entry site or via a splicing donor-acceptor mechanism.

The expression of the viral antigen or the immuno-stimulator may be controlled by a promoter homologous to the native progenitor of the

5  recombinant virus. Alternatively, the expression of the viral antigen may be controlled by a promoter heterologous to the native progenitor of the recombinant virus. For example, the promoter heterologous to the native progenitor of the recombinant virus may be a eukaryotic promoter such as insulin promoter, human cytomegalovirus (CMV) promoter and its early

10  promoter, simian virus SV40 promoter, Rous sarcoma virus LTR promoter/enhancer, the chicken cytoplasmic β-actin promoter, and inducible promoters such as the tetracycline-inducible promoter.

The pathogenic virus may be any pathogenic virus that causes pathogenic effects or disease in a host such as human, domestic animals or

15  other mammals. Thus, the recombinant virus can be used as a vaccine for protecting the host from infection of the pathogenic virus.

In a variation, the pathogenic virus may be various strains of human immunodeficiency virus (HIV), such as HIV-1 and HIV-2. The viral antigen may be a HIV glycoprotein (or surface antigen) such as HIV GP120 and

20  GP41, a capsid protein (or structural protein) such as HIV P24 protein, or other HIV regulatory proteins such as Tat, Vif and Rev proteins.

In another variation, the pathogenic virus may be influenza virus. The viral antigen may be an influenza glycoprotein such as influenza HA1, HA2 and NA.

25  In another variation, the pathogenic virus may be Ebola virus. The viral antigen may be an Ebola glycoprotein or surface antigen such as Ebola GP1 and GP2 protein.

In yet another variation, the pathogenic virus may be hepatitis virus such as hepatitis A, B, C, D or E virus. The viral antigen may be a surface

30  antigen or core protein of hepatitis A, B, C, D or E virus. For example, the viral antigen may be a surface antigen or core protein of hepatitis B virus such as the small hepatitis B surface antigen (SHBsAg) (also referred to as the Australia antigen), the middle hepatitis B surface antigen (MHBsAg) and the

large hepatitis B surface antigen (LHBsAg). The viral antigen may also be a surface antigen or core protein of hepatitis C virus such as NS3, NS4 and NS5 antigens.

5     In yet another variation, the pathogenic virus may be a respiratory syncytial virus (RSV). For example, the RSV viral antigen may be the glycoprotein (G-protein) or the fusion protein (F-protein) of RSV, for which the sequences are available from GenBank.

    In yet another variation, the pathogenic virus may be a herpes simplex virus (HSV) such as HSV-1 and HSV-2. For example, the HSV viral antigen

10     may be the glycoprotein D from HSV-2.

    In yet another variation, the viral antigen may be a tumor antigen or viral oncogene such as E6 and E7 of human papilloma virus, or cellular oncogenes such as mutated ras or p53.

    It is noted that, other virus-associated proteins or antigens are readily

15     available to those of skill in the art. Selection of the pathogenic virus and the viral antigen is not a limiting factor in this invention.

    The viral antigen may be a full-length antigenic viral protein or a portion of the antigenic viral protein that contains the predominant antigen, neutralizing antigen, or epitope of the pathogenic virus. Alternatively, the viral

20     antigen contains the conserved region of glycoproteins between at least two strains of the same pathogenic virus.

    In a variation, the viral antigen may be a modified antigen that is mutated from a glycoprotein of the pathogenic virus such that the viral antigen is rendered non-functional as a viral component but retains its antigenicity.

25     Such modification of the viral antigen includes deletions in the proteolytic cleavage site of the glycoprotein, and duplications and rearrangement of immunosuppressive peptide regions of the glycoprotein.

    The recombinant virus also expresses an immuno-stimulator to mimic cytokine-releasing response of a host cell upon viral infection and further

30     augments immune response to the viral antigen co-expressed from the recombinant virus. The immuno-stimulator may preferably be a cytokine. Examples of cytokine include, but are not limited to, interleukin-2, interleukin-4, interleukin-12, β- interferon, λ-interferon, =γ-interferon, granulocyte colony

stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF).

In another embodiment, a recombinant virus is provided for eliciting an immune response in a host infected by the virus. The recombinant virus

5   comprises: an antigen sequence heterologous to the recombinant virus that encodes a viral antigen from a pathogenic virus, expression of the viral antigen eliciting an immune response directed against the viral antigen and cells expressing the viral antigen in the host upon infection of the host by the recombinant virus.

10   According to this embodiment, the recombinant virus is preferably be replication-incompetent adeno-associated virus, SV40 virus, retrovirus, herpes simplex virus or vaccinia virus. The benign virus may preferably have the pathologic regions of the native progenitor of the benign virus deleted but retains its infectivity to the host.

15   Optionally, the recombinant virus includes an immuno-stimulator sequence heterologous to the recombinant virus that encodes an immuno-stimulator whose expression in the host enhances the immunogenicity of the viral antigen.

The present invention also provides genetic vaccines that elicit strong and long-lasting immune response to pathogenic bacteria. In one

20   embodiment, a recombinant virus is provided as a genetic bacteria vaccine for eliciting an immune response in a host infected by the recombinant virus. The viral genome of the recombinant virus comprises: a plurality of antigen sequences heterologous to the recombinant virus, each encoding a bacterial

25   antigen from a pathogenic bacteria, expression of the plurality of the bacterial antigen sequences eliciting an immune response directed against the bacterial antigen and cells expressing the bacterial antigen in the host upon infection of the host by the recombinant virus. The recombinant virus may preferably be replication-incompetent and not cause a malignancy naturally

30   associated with the pathogenic bacteria in the host.

The pathogenic bacteria may be any pathogenic bacteria that causes pathogenic effects or diseases in a host, such as *bacillus tuberculoses*, *bacillus anthracis*, and *spirochete Borrelia burgdorferi* that causes the Lyme

disease in animals. The plurality of antigen sequences may encode lethal factors, protective antigen, edema factors of the pathogenic bacteria, or combination thereof.

The present invention also provides parasites vaccines that elicit strong and long-lasting immune response to pathogenic parasites. In one embodiment, a recombinant virus is provided as a parasite vaccine for eliciting an immune response in a host infected by the benign virus. The recombinant virus comprises: a plurality of antigen sequences heterologous to the recombinant virus, each encoding a parasitic antigen from a pathogenic parasite, expression of the plurality of the parasitic antigen sequences eliciting an immune response directed against the parasitic antigen and cells expressing the parasitic antigen in the host upon infection of the host by the recombinant virus. The recombinant virus may preferably be replication-incompetent and not a cause malignancy naturally associated with the pathogenic parasite in the host.

The pathogenic parasite may be any pathogenic parasite that causes pathogenic effects or diseases in a host, such as malaria and protozoa such as Cryptosporidium, Eimeria, Histomonas, Leucocytozoon, Plasmodium, Toxoplasma, Trichomonas, Leishmania, Trypanosoma, Giardia, Babesia, and Theileria. The plurality of antigen sequences may encode coat proteins, attachment proteins of the pathogenic parasites, or combinations thereof.

The present invention also provides viral vaccines that present multiple antigens to the host to further mimic natural infection of a native pathogenic virus and induce strong and long-lasting immune response to various strains or types of the pathogenic virus in the host.

In one embodiment, a recombinant virus is provided as a viral vaccine for eliciting an immune response in a host infected by the virus. The recombinant virus comprises: a plurality of antigen sequences heterologous to the recombinant virus, each encoding a viral antigen from a pathogenic virus, expression of the plurality of the antigen sequences eliciting an immune response directed against the viral antigen and cells expressing the viral antigen in the host upon infection of the host by the recombinant virus. The

recombinant virus may preferably be replication-incompetent and not cause a malignancy naturally associated with the pathogenic virus in the host.

According to the embodiment, the recombinant virus may be any virus, preferably replication-incompetent adenovirus, adeno-associated virus, SV40

5    virus, retrovirus, herpes simplex virus or vaccinia virus. The recombinant virus may also preferably have the pathologic regions of the native progenitor of the benign virus deleted but retain its infectivity to the host.

Also according to the embodiment, the plurality of the antigen sequences may be multiple copies of the same antigen sequence or multiple

10    antigen sequences that differ from each another.

In a variation of the embodiment, at least two of the plurality of the antigen sequences are expressed from a promoter bicistronically via an internal ribosomal entry site or via a splicing donor-acceptor mechanism.

Alternatively, at least two of the plurality of the antigen sequences are

15    expressed from a promoter to form a fusion protein.

Also according to the embodiment, the recombinant virus further comprises at least one promoter heterologous to the native progenitor of the recombinant virus that controls the expression of at least two of the plurality of the antigen sequences. Examples of the promoter heterologous to the native

20    progenitor of the recombinant virus include, but are not limited to, insulin promoter, CMV promoter and its early promoter, SV40 promoter, Rous sarcoma virus LTR promoter/enhancer, the chicken cytoplasmic β-actin promoter, and inducible promoters such as tetracycline-inducible promoter.

Also according to the embodiment, the plurality of antigen sequences

25    may be a combination of antigens from at least two strains of the pathogenic virus.

Optionally, the plurality of antigen sequences may be a combination of antigens from at least two different pathogenic viruses. For example, the plurality of antigen sequences may be a combination of antigens from HIV-1,

30    HIV-2, herpes simplex virus type 1, herpes simplexvirus type 2, influenza virus, Marburg virus, Ebola virus, and hepatitis A, B, C, D, and E viruses.

In a variation of the embodiment, the viral genome of the recombinant virus may further comprise one or more immuno-stimulator sequences that is heterologous to the recombinant virus and encodes an immuno-stimulator whose expression in the host enhances the immunogenicity of the viral

5    antigen. For example, the immuno-stimulator may be a cytokine. Examples of the cytokine include, but are not limited to, interleukin-2, interleukin-4, interleukin-12, $\beta$-interferon, $\lambda$-interferon, $\gamma$-interferon, G-CSF, and GM-CSF.

According to the variation, the one or more immuno-stimulator sequences may be multiple copies of the same immuno-stimulator sequence

10    or multiple immuno-stimulator sequences that differ from each other.

Optionally, at least two of the immuno-stimulator sequences may be expressed from a promoter bicistronically via an internal ribosomal entry site or via a splicing donor-acceptor mechanism. Alternatively, at least two of the immuno-stimulator sequences may be expressed from a promoter to form a

15    fusion protein.

The DNA sequence encoding viral antigen(s) is inserted into any non-essential region of the replication defective virus. In the case of adenovirus, for example, the nucleic acid is preferably inserted into the E1, E3 and/or E4 region of the adenovirus and most preferably into the E4 region. Because the

20    E1, E3 and E4 regions are available as insertion sites, the present invention also contemplates separate insertion of more than one encoding sequence.

In the recombinant viral vector vaccines of the present invention, the selected nucleotide sequences of the viral antigens are operably linked to control elements that direct transcription or expression thereof in the subject

25    *in vivo*. Either homologous or heterologous viral control sequences can be employed. Useful heterologous control sequences generally include those derived from sequences encoding hostian or viral genes. Examples include, but are not limited to a cytomegalovirus (CMV) promoter such as the CMV immediate early promoter region ($CMV_{ie}$), SV40 early promoter, mouse

30    mammary tumor virus LTR promoter, adenovirus major late promoter (AdMLP), a herpes simplex virus promoter,and a retrovirus LTR promoter. Preferably, any strong constitutive promoter may be operatively linked to viral

antigens or cytokines. More preferably the viral promoter is CMV immediate early promoter (CMV$_{ie}$).

5

**Figures 1A-1C** illustrate a method for constructing a recombinant adenoviral vector as a genetic vaccine of the present invention. The recombinant adenoviral vector of the present invention is constructed by using shuttle plasmids or vectors carrying multiple antigen genes and multiple cytokine genes.

10

**Figure 1A** illustrates a shuttle plasmid (pLAd.Antigen) containing two antigen genes, Antigen 1 and Antigen 2.. The shuttle plasmid pLAd.Antigen contains the left end of the adenoviral genome including the left long terminal repeats L-TR, and an adenoviral packaging signal ($\psi$). The E1 region of the adenovirus is replaced by a multiple gene expression cassette and CMV$_{ie}$ promoter.

15

Genes encoding Antigen 1 and Antigen 2 are placed under the transcriptional control of the CMV$_{ie}$ promoter by a splicing mechanism at the SD and SA sites. The plasmid pLAd.Antigen also contains a SV40 polyadenylation site, as well as prokaryotic replication origin and ampicillin-resistance gene for DNA propagation in bacteria.

20

**Figure 1B** illustrates another shuttle plasmid (pRAd.Cytokines) containing multiple cytokine genes such as IL-2, INF, and IL-8. The shuttle plasmid pRAd.Cytokines contains the right end of the adenoviral genome including the right long terminal repeats R-TR. Most of the E4 region (except orf6) is replaced by the cytokine genes. Expression of cytokine genes is under the transcriptional control of the CMV$_{ie}$ promoter via an internal

25

ribosomal entry site (IRES) and by a splicing mechanism at the SD and SA sites. The plasmid pRAd.Cytokines also contains a bovine growth hormone (BGH) polyadenylation site, as well as a prokaryotic replication origin and ampicillin-resistance gene for DNA propagation in bacteria.

The recombinant adenoviral genome is assembled from the two shuttle

30

plasmids, pLAd.Antigen and pRAd.Cytokines, which carries the left and right end of the adenoviral genome, respectively. The shuttle plasmids pLAd.Antigen and pRAd.Cytokines are digested with restriction enzymes such

as XbaI and EcoRI, respectively.

As illustrated in **Figure 1C**, the fragments corresponding to the left end and right end of adenovirus from these two shuttle plasmids, pLAd.Antigen and pRAd.Cytokines, are isolated and ligated to the middle section of the

5    adenoviral genome (the adenovirus backbone).

The ligated vector genome DNA is then transfected into 293HK cells that express the E1 proteins of adenovirus. In the presence of E1 proteins, the vector genome in which the E1 has been deleted can replicate and be packaged into viral particle, i.e. producing the recombinant adenoviral vector

10    that can be used as a genetic vaccine of the present invention. The E1 region which is preserved in a native adenoviral genome but deleted from the recombinant viral genome is an example of the pathologic region native to the native progenitor of the recombinant virus: the wild type adenovirus.

**Figure 5** illustrates an example of a genetic vaccine constructed by

15    using the method described above. The replication defective adenovirus, type 5, is the vector backbone into which viral antigen and cytokines are inserted in the E1 region. The viral antigens are expressed using the CMVie promoter. The gene for the viral antigen is followed by the gene encoding INF-γ and GM-CSF, utilizing 2 IRES sequences to achieve expression of the

20    three proteins from a single mRNA. IL2 and IL4 are controlled by a second $CMV_{ie}$ promoter as a bi-cistronic cassette, followed by a second bi-cistronic cassette that express the two subunits of IL12 in the E4 region. Those skilled in the art will appreciate that the present invention is not limited to the structure discussed above, but that alternative cytokines may be used alone

25    or in combination with these and/or other cytokines. The detailed information about of these cytokines are described in the following section.

## 2.   Cytokines Co-Expressed With Viral Antigens

The recombinant virus of the present invention may also express an

30    immuno-stimulator to mimic cytokine-releasing response of a host cell upon viral infection and further augment immune response to the viral antigen co-expressed from the recombinant virus. The immuno-stimulator may be an

immunoenhancing cytokine to further stimulate the immune system. The recombinant virus may encode one or multiple cytokines in any combination. Alternatively, multiple cytokines may be expressed by more than one recombinant virus or delivered to the host by using other techniques such as

5    delivery via naked DNA plasmids or injection of cytokine proteins.

Examples of cytokine include, but are not limited to, interleukin-2, interleukin-4, interleukin-8, interleukin-12, $\beta$- interferon, $\lambda$-interferon, $\gamma$-interferon, granulocyte colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF).

10

Cytokines are immunodmodulatory molecules particularly useful in the vaccines of the invention as they are pleitropic mediators that modulate and shape the quality and intensity of the immune response. Cytokines are occasionally autocrines or endocrines, but are largely paracrine hormones

15    produced in nature by lymphocytes and monocytes.

As used herein, the term "cytokine" refers to a member of the class of proteins or peptides that are produced by cells of the immune system and that regulate or modulate an immune response. Such regulation can occur within the humoral or the cell mediated immune response and includes

20    modulation of the effector function of T cells, B cells, NK cells, macrophages, antigen presenting cells or other immune system cells.

Cytokines are typically small proteins or glycoproteins having a molecular mass of less than about 30 kDa. As used herein the term cytokine encompasses those cytokines secreted by lyphocytes andother cell types

25    (often designated as lymphokines) as well as cytokines secreted by monocytes and macrophages and other cell types (often designated as monokines). As used herein, the term cytokine encompasses those cytokines secreted by lymphocytes andother cell types as well as cyotkines secreted by monocytes and macrophages and other cell types. The term cytokine

30    includes the interleukins, such as IL-2, IL-4, IL-8 and IL-12, which are molecules secreted by leukocytes that primarily affect the growth and differentiation of hematopoietic and immune system cells. The term cytokine also includes hematopoietic growth factors and, in particular, colony

stimulating factors such as colony stimulating factor-1, granulocyte colony stimulating factor and granulocyte macrophage colony stimulating factor.

The cytokines can have the sequence of a naturally occurring cytokine or can have an amino acid sequence with substantial amino acid sequence

5   similarity, e.g., 60-95% amino acid sequence similarity, preferably 70-98% amino acid sequence, and most preferably 75-95% amino acid sequence similarity to the sequence of a naturally occurring cytokine.

Thus, it is understood that limited modifications to a naturally occurring sequence can be made without destroying the biological function of the

10  cytokine. For example, minor modifications of gamma interferon that do not destroy its function fall within the definition of gamma interferon. These modifications can be deliberate, as through site-directed mutagenesis, or can be accidental such as through mutation. The preferred cytokines are IL-2, IL-8, IL-12, or γ- interferon, β-interferon, λ-interferon, GM-CSF, or G-CSF or a

15  combination thereof.

Interleukin -2 is a lymphokine produced by helper T cells and is active in controlling the magnitude and type of the immune response. Smith, K. A., *Ann. Rev. Immunol.* 2, 319-333 (1984). Other functions have also been ascribed to IL-2 including the activation of NK cells (Minato, N. et al., *J. Exp.*

20  *Med.* 154, 750 (1983)) and the stimulation of cell division in large granular lymphocytes and B cells. Tsudo, M. et al.. *J. Exp. Med.* 160, 612-616 (1984). Studies in mice and humans have demonstrated that deficient immune responsiveness both *in vivo* and *in vitro* can be augmented by IL-2. For example, exogenous IL-2 can restore the immune response in

25  cyclophosphamide-induced immunosuppressed mice (Merluzzi, V. J. et al. *Cancer Res.* 41, 850-853 (1981)) and athymic (nude) mice. Wagner, H. et al. *Nature* 284, 278-80 (1982). Furthermore, IL-2 canrestore responsiveness of lymphocytes from patients with various immunodeficiency states such as leprosy and cancer. Vose, B. M. et al. *Cancer Immuno.* 13, 105-111 (1984).

30  The genes for murine (Yokota, T. et al. *Proc. Natl. Acad. Sci. USA* 82, 68-72 (1985)) and human (Taniguchi, T. et al. *Nature*, 302, 305-307 (1983)) IL-2 have been cloned and sequenced.

Interleukin-4 is a T cell derived factor that acts as an induction factor on resting B cells, as a B cell differentiation factor and as a B cell growth factors. Sevenusar, E. Eur. J. Immunol. 17, 67-72 (1987). The gene for human IL-4 has been isolated and sequenced. Lee, F. et al. Proc. Natl.

5 Acad. Sci. USA 83, 2061-2065 (1986).

IL-12 is a recently characterized heterodimeric cytokine that has a molecular weight of 75 kDa and is composed of disulfide-bonded 40 kDa and 35 kDa subunits. It is produced by antigen presenting cells such as macrophages, and binds to receptors on activated T, B and NK cells (Desai,

10 B. B., et al., *J. Immunol.*, 148:3125-3132 (1992); Vogel, L. A., et al., *Int. Immunol.*, 8:1955-1962 (1996)). It has several effects including 1) enhanced proliferation of T cells and NK cells, 2) increased cytolytic activities of T cells, NK cells, and macrophages, 3) induction of IFN- production and to a lesser extent, TNF-$\alpha$ and GM-CSF, and 4) activation of TH1 cells. (Trinchieri, G., et

15 al., *Blood*, 84:4008-4027 (1994). IL-12 has been shown to be an important costimulator of proliferation in Th1 clones (Kennedy et al., *Eur. J. Immunol.* 24:2271-2278 (1994)) and leads to increased production of IgG2a antibodies in serum (Morris, S. C., et al., *J. Immunol.* 152:1047-1056 (1994); Germann, T. M., et al., Eur. *J. Immunol.*, 25:823-829 (1995); Sher, A., et al., *Ann. N.Y.*

20 *Acad. Sci.*, 795:202-207 (1996); Buchanan, J. M., et al., *Int. Immunol.*, 7:1519-1528 (1995); Metzger, D. W. et al., *Eur. J. Imunol.*, 27:1958-1965 (1997)). Administration of IL-12 can also temporarily decrease production of IgG1 antibodies (Morris, S. C., et al., *J. Immunol.* 152:1047-1056 (1994); McKnight, A. J., *J. Immunol.* 152:2172-2179 (1994); Buchanan, J. M., et al.,

25 *Int. Immunol.*, 7:1519-1528 (1995)), indicating suppression of the Th2 response. The purification and cloning of IL-12 are disclosed in WO 92/05256and WO 90/05147, and in EP 322,827 (identified as "CLMF"). All of the above effects were observed in adult animals.

Interferons (IFNs) are relatively small, species-specific, single chain

30 polypeptides, produced by hostian cells in response to exposure to a variety of inducers such as viruses, polypeptides, mitogens and the like. They exhibit antiviral, antiproliferative and immunoregulatory properties and are, therefore,

of great interest as therapeutic agents in the control of cancer and various other antiviral diseases (J. Desmyter et al., *Lancet* 11, 645-647 (1976); R. Derynck et al., *Nature* 287, 193 (1980)). Human interferons are grouped into three classes based on their cellular origin and antigenicity: α-interferon

5    (leukocytes), β-interferon (fibroblasts) and γ-interferon (B cells). Recombinant forms of each group have been developed and are commercially available.

γ-interferon is also a T cell derived molecule which has profound effects on the immune response. The molecule promotes the production of immunoglobulin by activated B cells stimulated with interleukin-2. γ-interferon

10   also increases the expression of histocompatability antigens on cells which associated with viral antigens to stimulate cytotoxic T cells. The gene for human γ-interferon has been isolated and sequenced. Gray, P. W. et al., *Nature* 295, 503-508 (1982).

Human alpha interferons (also known as Leukocyte interferons)

15   comprise a family of about 30 protein species, encoded by at least 14 different genes and about 16 alleles. Some of these alpha interferon protein species have been shown to have antiviral, antigrowth and immunoregulatory activities. *See, e.g.*, Pestka et al., *Ann. Rev. Biochem.*, 56:727 (1987). The therapeutic efficacy of human alpha interferons has been established for

20   human cancers and viral diseases. For example, recombinant interferons (IFN alpha-2a, IFN alpha-2b, IFN alpha-2c), cell-line derived interferon (IFN alpha-n1) and interferon derived from leukocytes (IFN alpha-n3) are currently used for the treatment of Condyloma acuminata, hepatitis (Weck et al., *Am. J. Med.*, 85(Suppl 2A):159 (1988); Korenman et al., *Annal. Intern. Med.*,

25   114.:629 (1991); Friedman-Kien et al., *JAMA*, 259:533 (1988)), for the regression of some malignancies (Baron et al., *JAMA*, 266:1375 (1991)), for the treatment of AIDS related Kaposi's sarcoma (Physicians Desk Reference, 47th edit., eds. Medical Economics Data, Montvale, N.J., p. 2194 and 2006 (1993)) and are currently being considered for the treatment of human

30   acquired immunodeficiency syndrome (AIDS) either alone or in combination with other antiviral agents (Hirsch, *Am. J. Med.*, 85(Suppl 2A):182 (1988)).

β-interferon has been shown to be a glycoprotein by chemical measurement of its carbohydrate content. It has one N-glycosidyl attachment site (E. Knight, Jr., *Proc. Natl. Acad. Sci.*, 73, 520 (1976); E. Knight, Jr., and D. Fahey, *J. Interferon Res.*, 2 (3), 421 (1982)). Even though not much is

5    known about the kinds of sugars which make up the carbohydrate moiety of β-interferon, it has been shown that the carbohydrate moiety is not essential for its antigenicity, biological activity or hydrophobicity (T. Taniguchi et al., supra; E. Knight, Jr., supra; and E. Knight, Jr. and D. Fahey, supra). Beta-interferon can be induced in fibroblasts by viral challenge and contains about

10   165 amino acids. The sequence of  -interferon is known. Fiers et al. *Philos. Trnas. R. Soc. Lond., B, Biol. Sci.* 299:29-38 (1982).

GM-CSF is a cytokine important in the maturation and function of dendritic cells. It binds receptors on dendritic cells and stimulates these cells to mature, present antigen, and prime naive T cells. Dendritic cells form a

15   system of highly efficient antigen-presenting cells. After capturing antigen in the periphery, dendritic cells migrate to lymphoid organs and present antigens to T cells. These potent antigen-presenting cells are unique in their ability to interact with active naive T cells. The potent antigen-presenting capacity of dendritic cells may be due in part to their unique life cycle and high level

20   expression of major histocompatibility complex class I and II molecules and co-stimulatory molecules. The sequence of human GM-CSF is known. Wong et al., *Science* 228:810-815 (1985).

Granulocyte colony stimulating factor (G-CSF) is one of the hematopoietic growth factors, also called colony stimulating factors, that

25   stimulate committed progenitor cells to proliferate and to form colonies of differentiating blood cells. G-CSF preferentially stimulates the growth and development of neutrophils, and is useful for treating in neutropenic states. Welte et at., *PNAS-USA* 82: 1526-1530 (1985); Souza et at., *Science* 232: 61-65 (1986) and Gabrilove, J. *Seminars in Hematology* 26: (2) 1-14 (1989).

30   G-CSF increases the number of circulating granulocytes and has been reported to ameliorate infection in sepsis models. G-CSF administration also inhibits the release of tumor necrosis factor (TNF), a cytokine important to

tissue injury during sepsis and rejection. See, e.g., Wendel et al., *J. Immunol.*, 149:918-924 (1992). The cDNAs for human (Nagata et al., *Nature* 319;415, 1986) and mouse G-CSF (Tsuchiya et al., *PNAS* 83, 7633, 1986) have been isolated, permitting further structural and biological

5    characterization of G-CSF.

In humans, endogenous G-CSF is detectable in blood plasma. Jones et al., *Bailliere's Clinical Hematology* 2 (1): 83-111 (1989). G-CSF is produced by fibroblasts, macrophages, T cells trophoblasts, endothelial cells and epithelial cells and is the expression product of a single copy gene

10    comprised of four exons and five introns located on chromosome seventeen. Transcription of this locus produces a mRNA species which is differentially processed, resulting in two forms of G-CSF mRNA, one version coding for a protein of 177 amino acids, the other coding for a protein of 174 amino acids. Nagata et at., *EMBO J* 5: 575-581 (1986). The form comprised of 174 amino

15    acids has been found to have the greatest specific *in vivo* biological activity. G-CSF is species cross-reactive, such that when human G-CSF is administered to another host such as a mouse, Canine or monkey, sustained neutrophil leukocytosis is elicited. Moore et at. *PNAS-USA* 84: 7134-7138 (1987).

20    The present invention provides an effective means for enhancing the immune response to the specific foreign antigenic polypeptides of recombinant viruses. Although any foreign antigenic polypeptide can be used in the vaccine of the present invention, the vaccine is particularly useful in vaccines against the HIV virus and the Ebola virus, since these viruses have

25    a negative effect on the host's immune system. The vaccine is also very useful for immunization against hepatitis B and C virus.

### 3.    Genetic Vaccines Against HIV Infection

The genetic vaccine of the present invention also addresses the need
30    for an efficient vaccine against the HIV virus. According to the present invention the genetic vaccine may be a recombinant benign virus in which the viral genome carries one or more antigens from HIV, such as HIV glycoproteins (e.g. GP120 and GP41) or capsid proteins (e.g. P24).

Sequences of these HIV antigens may be modified such as deletion of the immunosuppressive regions of the HIV glycoproteins.

The HIV virus causes the disease known as Acquired Immune Deficiency Syndrome (AIDS). AIDS has been described as a modern plague

5 since its first description in 1981, it has claimed over 60,000 victims, and accounted for over 32,000 deaths in the United States alone. The disease is characterized by a long aysmptomatic period followed by a progressive degeneration of the immune systemand the central nervous system. The virus may remain latent in infected individuals for five or more years before

10 symptoms appear, and thus, the true impact of the disease has yet to be felt. Many Americans may unknowingly be infected and capable of infecting others who might come into contact with their body fluids. Thus, if unchecked, the personal, social and economic impact of AIDS will be enormous.

15 The HIV virus is a retrovirus. Thus, its genetic matierial is RNA, which encodes the information for viral replication. Upon infection of a host cell, the RNA acts as a template for the transcription to DNA, which is catalyzed by an enzyme called reverse transcriptase. The DNA so produced enters the cell nucleus where it is integrated into the host DNA as a provirus. When properly

20 activated, the retroviral-derived DNA is transcribed and translated to produce RNA containing virions, which are then released from the cell by a budding process.

When an individual becomes infected with HIV, the virus preferentially attaches to and enters a particular class of white blood cells, called T4

25 lymphocytes, which are characterized by the presence of a cell surface marker termed CD4. These white blood cells play an integral role in the immune system, functioning as critical components of both the humoral and cellular immune response. Much of the deleterious effect of HIV can be attributed to the functional depression or destruction of T4 lymphocytes.

30 The intact HIV virion is roughly spherical and is approximately110 nm in diameter. The virion has an outer membrane covered with spike-like structures made up of glycoprotein, gp160/120. In addition, there exists a transmembrane protein termed gp41. Inside the virion are two structural

proteins: an outer shell composed of the phosphoprotein, p17, and an inner nucleoid or central core made up of the phosphoprotein, p24. The viral RNA is present inside the core along with two copies of the reverse transcriptase enzyme, p66/51, which is necessary for the synthesis of viral DNA from the

5    RNA template. The HIV RNA genome encodes three major structural genes: gag, pol and env, which are flanked at either end by long terminal repeat (LTR) sequences. The gag gene codes for the group-specific core proteins, p55, p39, p24, p17 and p15. The pol genes code for the reverse transcriptase, p66/p51, and the protease, p31. The env genes encode the

10   outer envelope glycoprotein, gp120, and its precursor, gp160, and the transmembrane glycoprotein, gp41. Some of the genes tend to be highly variable, particularly the env genes. In addition, there are five other genes, not shared by other retroviruses, which are either involved in transcriptional or translational regulation or encode other structural proteins. The entire HIV

15   genome has now been sequenced. *See* Ratner et al. Nature 313:277 (1985), which is incorporated herein by reference.

       The HIV envelope protein has been extensively described, and the amino acid and RNA sequences encoding HIV envelope from a number of HIV strains are known. *See* Myers, G. et al., *Human Retroviruses and AIDS:*

20   *A compilation and analysis of nucleic acid and amino acid sequences*, Los Alamos National Laboratory, Los Alamos, N.M. (1992). The *env* genes of various strains of HIV are predicted to encode proteins of 850 to 880 amino acids. Extensive glycosylation of the Env precursor polyprotein during synthesis produces gp160 (about 160 kilodaltons) which is also the major

25   form of the *env* gene product detected in infected cells. Gp160 forms a homotrimers and undergoes glycosylation with the Golgi apparatus.

       The functional domains of gp160 includes, starting from N-terminus, Signal peptide, Variable regions 1 through 5 which encompass CD4 binding sites (e.g., $Thr^{257}$, $Trp^{427}$, $Asp^{368}/Glu^{370}$, and $Asp^{457}$), Proteolytic processing

30   site (also called the cleavage site between gp120 and gp41), Fusion domain, Leucine zipper motif, transmembrane domain, and Lentivirus lytic peptides (LLP) 1 and 2. Although the nucleotide and amino acid sequences of gp120 and the numbering thereof from various isolates and strains of HIV may differ,

the region encoding the functional domains can be readily identified by the teaching in Luciw (1996) in "Fundamental Virology", 3$^{rd}$ ed., eds., Fields et al., Lippincott-Raven Publishers, Philadelphia, Chapter 27, pp. 845-916.

5         The signal peptide at the N-terminus of the Env precursor gp160 directs ribosomes translating the nascent protein to the endoplasmic reticulum; an intracellular proteinase removes this signal peptide during Env gp biogenesis. The Env precursor gp160 is cleaved at the processing site by a cellular protease to produce gp120 (designated SU subunit) and gp41 (designated TM subunit). Gp120 contains most of the external, surface-

10 exposed, domains of the envelope glycoprotein complex. Gp41 contains a transmembrane domain and remains in a trimeric configuration, and it interacts with gp120 in a non-covalent manner. The subunits of gp41 include: Fusion peptide, Leucine zipper-like region, transmembrane domain (TM), LLP1 and LLP2.

15         The gp120 subunit contains five variable regions and six conserved regions. The variable (V) domains and conserved (C) domains of gp120 are specified according to the nomenclature of Modrow et al. (1987) "Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: predictions of antigenic epitopes in

20 conserved and variable regions", J. Virol. 61:570-578.

        The gp120 molecule consists of a polypeptide core of 60,000 daltons, which is extensively modified by N-linked glycosylation to increase the apparent molecular weight of the molecule to 120,000 daltons. The positions of the 18 cysteine residues in the gp120 primary sequence, and the positions

25 of 13 of the approximately 24 N-linked glycosylation sites in the gp120 sequence are common to all gp120 sequences. The hypervariable domains contain extensive amino acid substitutions, insertions and deletions. Sequence variations in these domains result in up to 30% overall sequence variability between gp120 molecules from the various viral isolates. Despite

30 this variation, all gp120 sequences preserve the virus's ability to bind to the viral receptor CD4 and to interact with gp41 to induce fusion of the viral and host cell membranes.

The HIV virus attaches to host cells by an interaction of the envelope glycoproteins with a cell surface receptor. It appears that when HIV makes contact with a T4 cell, gp120 interacts with the CD4 receptor. Recently, the crystal structure of the core domain of HIV-1 gp120 (strain HXB-2, a clade B

5      virus) has been solved by complexing the protein with a fragment of human CD and an antigen-binding fragment from a virus-neutralizing antibody that blocks chemokine-receptor binding. Kwong et al. (1998) "Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody", Nature 393:648-659. These studies revealed

10    that the gp120 core has a unique molecular structure that comprises two domains—an "inner domain" (which faces gp41) and an "outer" domain (which is mostly exposed on the surface of the oligomeric envelope glycoprotein complex). The two gp120 domains are separated by a "bridging sheet" that is not part of either domain. Binding to CD4 causes a

15    conformational change in gp120 which exposes the bridging sheet and may move the inner and outer domains relative to each other. It was also found that most of the carbohydrate molecules which are added to gp120 are added to the outer domain. This is consistent with the idea that that virus uses carbohydrate molecules to mask external antigenic epitopes on gp120.

20          Gp120 not only binds to the cellular CD4 receptor but also to HIV coreceptors such as the cellular chemokine receptors (e.g. CCR5). Upon binding to the receptor and/or coreceptor, the viral envelope is then fused with the cell membrane and the inner core of the virus enters the infected cell where the transcription of RNA into a DNA provirus is catalyzed by reverse

25    transcriptase. The provirus may remain in the cell in a latent form for some months or years, during which time the infected individual is asymptomatic. However, if the virus is later activated causing viral replication and immuno-suppression the individual will than be susceptible to the opportunistic infections associated with AIDS.

30          In one embodiment of the HIV vaccine of the present invention, a recombinant virus is provided for eliciting strong immune response against infection of HIV. The recombinant virus comprises: an antigen sequence heterologous to the recombinant virus that encodes an antigen from human

immunodeficiency virus (HIV), expression of the HIV antigen eliciting an immune response directed against the HIV antigen and cells expressing the HIV antigen in a host upon infection of the host by the recombinant virus; and an immuno-stimulator sequence heterologous to the recombinant virus that

5   encodes an immuno-stimulator whose expression in the host enhances the immunogenicity of the HIV antigen.   In a preferred embodiment, the recombinant virus is replication-incompetent and does not cause a malignancy naturally associated with HIV in the host.  The recombinant virus is used as a genetic vaccine to be administered to a host to induce or elicit

10  strong and long-lasting immunity against HIV infection.

In comparison with other approaches for developing HIV vaccine using denatured or attenuated HIV virion, the approach of the present invention should be safer and more efficient in eliciting strong immune response but not creating risks of reactivation of HIV, probably through recombination with the

15  wild type HIV infecting the host.

According to the present invention, the HIV antigen expressed by the genetic vaccine may be any antigen derived from a HIV virus, such as HIV surface, core/capsid, regulatory, enzyme and accessory proteins.  Examples of HIV surface protein include, but are limited to the products of the *env* gene

20  such as gp120 and gp41.  Examples of HIV capsid protein include, but are limited to the products of the *gag* gene such as the cleavage products of the Pr55$^{gag}$ by the viral encoded protease PR: the mature capsid proteins MA (p17), CA (p24), p2, NC (p7), p1 and p6.  Herderson et al. (1992) J. Virol. 66:1856-1865.  Examples of viral regulatory proteins include, but are not

25  limited to the products of the *tat* and *rev* genes: Tat and Rev.  Examples of viral enzyme proteins include, but are not limited to the products of the *pol* gene: p11 (protease or PR), p51 (reverse transcriptase or RT), and p32 (integrase or IN).  Examples of viral accessory proteins include, but are not limited to the products of the *vif, vpr, vpx, vpu* and *nef* genes: Vif, Vpr, Vpx,

30  Vpu and Nef.

In one embodiment, HIV Nef protein may serve as the HIV antigen expressed by the recombinant virus of the present invention.  For example, sequence encoding Nef (e.g., the *nef* sequence at position 8152-8523 for

BH10 strain of HIV and at position 8787-9407 for pNL4-3 strain of HIV) may be inserted into the vector.

In another embodiment, HIV Rev protein may serve as the HIV antigen expressed by the recombinant virus of the present invention. For example, 5 sequence encoding Rev (e.g., the *rev1* sequence at position 5969-6044 and the *rev2* sequence at position 8369-8643 for pNL4-3 strain of HIV) may be inserted into the vector.

In yet another embodiment, full length HIV Gag protein may serve as the HIV antigen expressed by the recombinant virus of the present invention. 10 For example, sequence encoding full leng Gag (e.g., the *gag* sequence at position 112-1650 for BH10 strain of HIV and at position 790-2292 for pNL4-3 strain of HIV) may be inserted into the vector.

Alternatively, capsid protein from HIV Gag protein (e.g. p24 CA) may serve as the HIV antigen expressed by the recombinant virus of the present 15 invention. For example, sequence encoding p24CA (e.g., the sequence at position 1186-1878 for BH10 strain of HIV and at position 508-1200 for pNL4-3 strain of HIV) may be inserted into the vector.

In yet another embodiment, the HIV antigen expressed by the recombinant virus is derived from the *env* gene products. For example, the 20 antigen is derived from the Env protein.

According to the embodiment, modifications or mutagenesis may be used to delete or mutate in certain region(s) of Env to render it non-functional and yet still contains neutralizing epitopes for its natural genicity. For example, the proteolytic processing site of Env may be deleted or mutated to 25 render it resistant to cleavage by cellular protease to produce gp120 and gp41 fragments. Deletion or mutation may also be carried out on the transmembrane and cytoplasmic domains of gp41 such as the TM, LLP-1 and LLP-2 domains. Compared to the wild type Env, the mutated Env protein should have a reduced risk of being incorporated into a wild type HIV that 30 infects the host and being exploited by HIV in its furtherance of the goal: destruction the host's immune system.

For example, wildtype HIV Env can be modified in the following ways. Wildtype gp120 sequence from BH10 strain of HIV and containing Env, Tat,

and Rev coding sequences can be digested with restriction enzymes EcoR I and Xho I to produce a fragment starting from nucleotide 5101 and ending at nucleotide 8252. The cytosolic domain of Env can be removed by deleting nucleotides from the coding sequence at position 7848-8150 for BH10 strain,

5  and 8610-8785 for pNL4-3 strain of HIV. The cleavage site of Env can be removed by deleting 12 nucleotides encoding amino acid sequence REKR at position 7101-7112 for BH10 strain, and 7736-7747 for pNL4-3 strain of HIV.

Also according to this embodiment, the modified Env protein may contain deletions in the regions that do not contain neutralizing epitopes. For

10  example, the V1 and V5 domains of gp120 may be deleted without sacrificing the natural antigenicity of gp120. Portions of the V2 and V3 domains of gp120 that do not contain neutralizing epitopes may also be deleted. Although the principle neutralizing domain (PND) has been found in the V3 domain, V2 and C4 domains of gp120 have also been found to contain

15  neutralizing epitopes. Among various strains or clades of HIV, the amino acid sequences of the neutralizing epitopes may be variable. However, it has been found that the amount of variation is highly constrained. Thus, the sequences not containing the neutralizing epitopes should be readily determined.

20  For example, sequence encoding V1 region of Env can be deleted at position 5961-6032 for BH10 strain, and 6602-6673 for pNL4-3 strain of HIV. Sequence encoding V2 region of Env can be deleted at position 6060-6161 for BH10 strain, and 6700-6796 for pNL4-3 strain of HIV. Optionally, sequence encoding both V1 and V2 regions of Env can be deleted at position

25  5961-6161 for BH10 strain, and 6602-6796 for pNL4-3 strain of HIV.

Alternatively, the HIV antigen expressed by the recombinant virus may be a subunit of gp120 which contains one or more selected variable (V) and/or conserved (C) domains. For example, the HIV antigen may be a gp120 subunit containing V2, V3 and C4 domains, or V3 and C4 domains.

30  The location of neutralizing epitopes in the V3 domain is well known. It has been found that neutralizing epitopes in the V2 and C4 domains are located between residues 163 and 200 and between about 420 and 440, respectively. In addition, residues for antibody binding also include residues 171, 174,

177, 181, 183, 187, 188 in the V2 domain and residues 429 and 432 in the C4 domains. Berman et al. (1999) Virology 265:1-9; and Berman (1998) AIDS Res. Human Retroviruses 15:115-132.

In another embodiment, the HIV antigen expressed by the recombinant
5  virus of the present invention may be a modified Env protein that contains deletions and/or mutations in the glycosylation sites. The gp120 of HIV-1 contains 24 potential sites for N-linked glycosylation (Asn-X-Ser/Thr); about 13 of the 24 glycosylation motifs are conserved in the different viral isolates. Analysis of HIV-1 Env gp proteins has demonstrated that 17 of 24 potential
10  glycosylation sites are modified with carbohydrate side chains. Mizuochi et al. (1990) J. Biol. Chem. 265:8519-8524; and Leonard et al. (1990) J. Biol. Chem. 265:10373-10382. Because of the extensive glycosylation of Env gp proteins, very few regions of the peptide backbone of gp120 protrude from the carbohydrate mass. Some of the glycosylation sites have been found in
15  non-neutralizing epitopes that dilute the immunity against true neutralizing epitopes or serve as decoy epitopes. Thus, deletion or mutation of these glycosylation sites may enhance immunity of the antigen by unmasking the true neutralizing epitopes.

In another embodiment, the different HIV antigens may be expressed
20  by the same recombinant virus of the present invention. For example, both Env, Tat and Rev proteins may be expressed from the same promoter such as a CMV early promoter via a retroviral splicing donor-acceptor mechanism. Optionally, HIV Gag protein, either in full length or a truncated or modified form (e.g., capsid protein p24), may also be expressed together with other
25  HIV antigens such as Env, Tat and Rev. Further, these HIV antigens may be expressed together with the immuno-stimulator(s) (e.g., IL-2, IL-12, INF-$\gamma$, and GMCSF) in single or multiple copies by the same recombinant viral vector.

For example, the sequences encoding the HIV antigens may be inserted into E1 region of an adenoviral vector and expressed from a CMV
30  early promoter via a retroviral splicing donor-acceptor mechanism or an IRES mechanism. The sequences encoding the immuno-stimulators may be inserted into E4 region of the same adenoviral vector and expressed from

another CMV early promoter via a retroviral splicing donor-acceptor mechanism or an IRES mechanism.

In yet another embodiment, the sequence encoding the HIV antigen in the recombinant virus of the present invention is a mosaic antigen that

5   contains sequences from different strains, isolates and/or clades of HIV viruses. A strain of HIV is the HIV isolated from an individual (an isolate), characterized and given a strain name (e.g., MN, LAI). Because of the heterogenecity of HIV, not two isolates are exactly the same. A group of related HIV isolates are classified according to their degree of genetic

10   similarity such as of their envelop proteins. There are currently two groups of HIV-1 isolates, M and O. The M group consists of at least 9 clades (also called subtypes), A through I. The O group may consist of a similar number of clades. Clades are genetically distinct but are all infectious. It is believed that by using a mosaic HIV antigen in the design of the genetic vaccine of the

15   present invention the vaccine produced should have an enhanced ability to stimulate the production of anti-HIV antibodies and HIV-specific cytotoxic T lymphocytes (CTLs) against a wider spectrum of "wild type" HIV strains.

In one embodiment, the mosaic HIV antigen in the recombinant virus contains antigens from multiple clades of HIV-1, including clade A (Accession

20   No: HIV-1 92UG037WHO.0108HED), B (Accession No: pNL4-3), C (Accession No: HIV-1 92BR025WHO.109HED), D (Accession No: HIV-1 92UG024.2), E (Accession No: HIV-1 93TH976.17), F (Accession No: HIV-1 93BR020.17), and G (Accession No: HIV-1 92RU131.9). Optionally, multiple repeats of restriction fragments of HIV antigen (e.g., Ava I fragments) from

25   different clades may be linked head-to-tail to generate an even more complex mosaic HIV antigen.

For example, an adenoviral vector may be constructed to the V3 loops of multiple clades as the mosaic HIV antigen. Optionally, HIV antigens with gp41 deletion from multiple clades may serve as the mosaic HIV antigen.

30   Alternatively, HIV antigens from multiple clades with V1 and V2 loops deleted from clade B (pNL4-3) may serve as the mosaic HIV antigen.

Yet optionally, a human gene Thy-1 GPA anchor sequence encoding amino acid sequence SWLLLLLLSLSLLQATDFMSL [SEQ ID NO: 9] may be added to the recombinant viral construct.

In another embodiment, the mosaic HIV antigen contains an Env
5    protein which comprises variable and constant domains of gp120 derived from different strains, isolates and/or clades of HIV viruses. For example, V2 domain from clade B of the M group may be mixed with V3 and C4 domains from clade C of the O group to generate a mosaic HIV antigen. Vaccination of individuals with such a mosaic antigen may stimulate CTLs with cross-
10   clade activity. In another word, these CTLs can recognize and kill target cells infected HIV from different clades.

Alternatively, the recombinant virus may express a plurality of HIV antigens, each of which is an antigen from a different strain, isolate or clade of HIV. For example, *env* genes from different clades of HIV can be cloned
15   into the recombinant virus and expressed in tandem to produces various Env proteins from these clades in the host cells. It is believed that expressing various Env proteins from different strains, isolates or clades of HIV in the host cells should enhance the ability of the genetic vaccine of the present invention to stimulate the production of anti-HIV antibodies and HIV-specific
20   cytotoxic T lymphocytes (CTLs) against a wider spectrum of "wild type" HIV strains. The host vaccinated with such a vaccine would be able to be immunized from infection of various strains of HIV.

By using the genetic vaccine of the present invention, individuals not infected by HIV may be immunized against HIV. For HIV-infected individuals
25   the vaccine may also be used boost their immune response and help fight against this virulent virus. Since the genetic vaccine can express high level of antigens and/or a variety of HIV glycoproteins and capsid proteins simultaneously, the vaccinated individuals should be immunized against various strains of HIV, such as HIV-1 and HIV-2. Additionally, since the
30   genetic vaccine can express high levels of cytokines to mimic the body's response to natural viral infection, the body's immune response to such a genetic vaccine against HIV should be strong and long-lasting, thereby achieving a life-long immunity against this deadly virus.

### 4. Genetic Vaccines Against Hepatitis Viruses

The genetic vaccine of the present invention also addresses the need for an efficient vaccine against hepatitis viruses such as hepatitis A, B, C, D, and E viruses. According to the present invention the genetic vaccine may be a recombinant benign virus in which the viral genome carries one or more antigens from a hepatitis virus, such as glycoproteins and core proteins of the hepatitis virus. Sequences of these HIV antigens may be modified such as deletion of the pathogenic regions of the hepatitis glycoproteins or coreproteins.

In particular, the recombinant virus of the present invention can be used as a vaccine to immunize individuals against Hepatitis B infections. Viral hepatitis B is caused by the Hepatitis B virus (HBV). HBV is estimated to have infected 400 million people throughout the world, making HBV one of the most common humanpathogens. Hepatocellular carcinomas (HCC), one of the most common cancers afflicting humans, is primarily caused by chronic HBV infection.

HBV is a mostly double-stranded DNA virus in the *Hepadnaviridae* family. The HBV genome is unique in the world of viruses due to its compact form, use of overlapping reading frames, and dependence on a reverse-transcriptase step, though the virion contains primarily DNA. The HBV genome has four genes: pol, env, pre-core and X that respectively encode the viral DNA polymerase, envelope protein, pre-core protein (which is processed to viral capsid) and protein X. The function of protein X is not clear but it may be involved in the activation of host cell genes and the development of cancer.

The diagnosis of HBV infection is generally made on the basis of serology. Virtually all individuals infected with HBV will have detectable serum hepatitis surface antigens (HBsAg). Despite notable successes of vaccines against HBV infection, it is still an on-going task. A review on modern hepatitis vaccines, including a number of key references, may be found in the Eddleston, *The Lancet*, p. 1142, May 12, 1990. *See also Viral Hepatitis and Liver Disease*, Vyas, B. N., Dienstag, J. L., and Hoofnagle, J.

H., eds., Grune and Stratton, Inc. (1984) and *Viral Hepatitis and Liver Disease*, Proceedings of the 1990 International Symposium, eds F. B. Hollinger, S. M. Lemon and H. Margolis, published by Williams and Wilkins.

5    According to the present invention, the viral antigen may be a surface antigen or core protein of hepatitis B virus such as the small hepatitis B surface antigen (SHBsAg) (also referred to as the Australia antigen), the middle hepatitis B surface antigen (MHBsAg) and the large hepatitis B surface antigen (LHBsAg).

10   Antigens of different types of HBV, such as Asian type C and America type A, may be expressed by the recombinant virus to elicit immune response to these types of HBV. The HBV surface antigen (HBsAg) or the core antigen (HBcAg) may be expressed by the recombinant virus of the present invention, separately or in combination (HBsAg + HBcAg).

15   For example, the sequences encoding multiple HBV antigens may be inserted into E1 or E4 region of an adenoviral vector and expressed from a CMV early promoter via a retroviral splicing donor-acceptor mechanism or an IRES mechanism. Further, these HBV antigens may be expressed in combination with one or more immuno-stimulators such as IL-2, IFN-$\gamma$ and GMCSF in single or multiple copies. Sequences encoding these cytokines

20   may be inserted into E4 or E1 region that is not occupied by the antigen sequences and expressed from another CMV early promoter via a retroviral splicing donor-acceptor mechanism or an IRES mechanism.

Specific combinations of inserts include, but are not limited to, HBsAg + HBcAg; HBsAg + HBcAg + IL-2; HBsAg + HBcAg + IFN-$\gamma$ + GMCSF; and

25   HBsAg + IFN-$\gamma$ + IFN-$\gamma$ + GMCSF.

The sequences encoding the immuno-stimulators may be inserted into E4 region of the same adenoviral vector and expressed from another CMV early promoter via a retroviral splicing donor-acceptor mechanism or an IRES mechanism.

30   Also according to the present invention, the viral antigen may be a surface antigen or core protein of hepatitis C virus such as NS3, NS4 and NS5 antigens.

For example, sequence(s) encoding the HCV antigen(s) may be inserted into E1 or E4 region of an adenoviral vector and expressed separately or in combination with one or more immuno-stimulators such as IL-2, IL-12, IFN-$\gamma$ and GMCSF in single or multiple copies.

Specific combinations include, but are not limited to,

(1) HCV wildtype E2 + wildtype E1;

(2) core of HCV;

(3) HCV E2 + E1 + core;

(4) HCV E2 + E1 + core + IL-2;

(5) HCV E2 + E1 + core + IL-2 + IFN-$\gamma$ + GMCSF; and

(6) HCV E2 + E1 + core + IL-2 + IFN-$\gamma$ + IL-12.

In another embodiment, multi copies of hypervariable regions (HVR) of HCV E1 and E2, e.g., five copies of HVR (5xHVR), may serve as the viral antigen in the recombinant virus, and may be expressed alone or in combination with one or more immuno-stimulators such as IL-2, IL-12, IFN-$\gamma$ and GMCSF in single or multiple copies.

Specific combinations include, but are not limited to,

(1) E2-5xHVR + E1;

(2) E2-5xHVR + E1 + IL-2;

(3) E2-5xHVR + E1 + core + IL-2;

(4) E2-5xHVR + E1 + core + IL-2 + IFN-$\gamma$ + GMCSF; and

(5) E2-5xHVR + E1 + core + IL-2 +IL-12.

By using the genetic vaccine of the present invention, non-hepatitis-infected individuals may be immunized against hepatitis virus. For hepatitis virus-infected individuals the vaccine may also be used boost their immune response and help fight against the hepatitis virus. Since the genetic vaccine can express high level of antigens and/or a variety of hepatitis glycoproteins and coreproteins simultaneously, the vaccinated individuals should be immunized against various strains and/or types of hepatitis virus, such as hepatitis A, B, C, D, and E virus. Additionally, since the genetic vaccine can

express high levels of cytokines to mimic the body's response to natural viral infection, the body's immune response to such a genetic vaccine against hepatitis should be strong and long-lasting, thereby achieving a life-long immunity against the hepatitis virus.

5

### 5.    Genetic Vaccines Against Ebola Virus

The genetic vaccine of the present invention also addresses the need for an efficient vaccine against the deadly virus, Ebola virus. According to the present invention the genetic vaccine may be a recombinant benign virus in
10    which the viral genome carries one or more antigens from Ebola hepatitis, such as glycoproteins (e.g. GP1 and GP2) of Ebola virus. Sequences of these Ebola antigens may be modified such as deletion of the immunosuppressive regions and/or other pathogenic regions of the Ebola virus.

Ebola virus is one of the most lethal viruses known to mankind with a
15    mortality rate of up to 90%. Johnson, K.M., *Ann Intern Med* 91(1):117-9 (1979). Victims of Ebola virus infection are subjected to a horrible hemorrhagic diseases which kills in a matter of days. The natural reservoir of the virus remains unknown, as do the specifics of pathogenesis of the infection. The virus has a very specific tropism for liver cells and cells of the
20    reticuloendothelial system, such as macrophages. Massive destruction of the liver is hallmark feature of the disease.

Although Ebola virus infection is rare, there is concern by public health officials about the potential for the disease to become an international epidemic as the Ebola virus is easily transmitted through human contact and
25    is extremely contagious. Outbreaks like those that have recently occurred in Africa could happen in industrialized countries due to the rapid and extensive nature of modern travel. Recent cases of Ebola virus infection in Africa send strong warnings to be prepared for the outbreaks of this extremely dangerous infectious disease. In addition, Ebola virus has a terrifying potential if used as
30    a biological weapon byterrorist nations or organizations. As in most cases of viral infection, the best approach to prevent an outbreak of Ebola virus is through vaccination. However, there currently is no effective vaccine nor treatment available against Ebola virus infection.

Ebola viruses are enveloped, negative strand RNA viruses, which belong to the family Filoviridae. There are three strains of filoviruses: Ebola, Marburg and Reston. The Ebola virus can enter the body a number of different ways such as an opening through which air is taken in because the virus can travel on airborne particles and it can also enter the body through any opening in the skin, such as cuts.

The Ebola virus has a non-segmented RNA genome that encodes all the viral structural proteins (nucleoprotein, matrix proteins VP24 and VP40), non-structural proteins (VP30, VP35) and viral polymerase. Peters, C.J., *West J Med* 164(1):36-8 (1996). Among the viral proteins, the envelope glycoproteins (GP) exist in two forms, a secreted glycoprotein (50-70 kDa) and a transmembrane glycoprotein (130-170 kDa) generated by transcriptional editing. Sanchez, A. et al., *Proc Natl Acad Sci U.S.A.*, 93(8):3602-7 (1996). Although the two forms of GP share 295 amino acid homology, they have distinct binding specificities, suggesting that they play different roles in the course of viral infection. The secreted glycoprotein (sGP) is the predominant form synthesized and secreted by the infected cells. It may play a role in suppressing the host immune system (Yang,Z., et al., *Science* 279(5353):1034-7 (1998)) and may serve as a decoy to allow the virus particle to escape from neutralizing antibodies, since the two forms of GPs partly share their antigenicity. Analysis of monoclonal antibodies from the human survivors of Ebola virus Zaire infection has revealed that the vast majority of them were specific to the sGP, and only a few bound weakly to GP. Maruyama, T., et al., *J Infect Dis*, 179 Suppl 1:S235-9 (1999), Maruyama, T., et al., *J Virol*, 73(7):6024-30 (1999). Although the exact mechanism by which the sGP may suppress the immune system is not clearly understood, the large amounts of sGP synthesized in the early phase of the infection are probably responsible for the inhibition of neutrophil infiltration of the infected sites (Yang, Z., et al., *Science* 279(5353):1034-7 (1998)) and the absence of humoral immune response in Ebola virus infected patients. Baize, S., et al., *Nat Med,* 5(4):423-6 (1999). This protein may also act to over-activate many types of immune cells which can lead to massive intravascular apoptosis - essentially a shut-down of the immune system.

Baize, S., et al., *Nat Med,* 5(4):423-6 (1999). The importance of the sGP to the Ebola virus life-cycle is also suggested by the fact it is present in all Ebola virus strains examined to date. Feldmann, H., et al., *Arch Virol Suppl,* 15:159-69 (1999).

5      The membrane glycoproteins are responsible for the attachment and penetration of the virions into target cells by mediating receptor binding and viral-cellular membrane fusion. Wool-Lewis, et al., *J. Virol,* 72(4):3155-60 (1998), Ito H., et al., *J. Virol,* 73(10):8907-12 (1999). They are synthesized as a single peptide precursor and cleaved by cellular enzymes (furin or cathepsin

10    B) into the two mature forms, GP1 and GP2. The two GPs remain associated through a disulfide bond linkage and remain anchored in the viral membrane by a transmembrane (TM) domain. Ito H., et al., *J. Virol,* 73(10):8907-12 (1999); Malashkevich, V.N., et al., *Proc Natl Acad Sci U.S.A.,* 96(6):2662-7 (1999). The proteolytic cleavage site is composed of 4-5 basic amino acid

15    residues that are similar to those found in the GPs of retrovirus, influenza, and paramyxoviruses. Garten, W., et al., *Biochimie,* 76(3-4):217-25 (1994). The cleavage event is essential for viral infectivity and is likely carried out by the same enzymes that cleave GPs of retrovirus or influenza viruses. Garten, W., et al., *Biochimie,* 76(3-4):217-25 (1994); Volchkov, V.E., et al., *Virology,*

20    245(1):110-9 (1994). In addition, Ebola virus GP may share a common mechanism of mediating viral infection with retroviral and influenza glycoproteins. Weissenhorn, W., et al., *Mol Membr Biol,* 16(1):3-9 (1999). Because membrane-bound GPs play critical roles in initiating virus infection and are also the predominant proteins exposed on the surface of the virions,

25    they are the primary targets for neutralizing antibodies against the virus.

One of the properties of Ebola viruses that make them lethal to the host is their ability to suppress the host immune system. Serologic analysis of patients who died of the Ebola virus infection showed no signs of humoral or cellular immune responses. Baize, S., et al., *Nat Med,* 5(4):423-6 (1999).

30    In contrast, antibodies against viral proteins and virus-specific T-cell activities were detected in a few survivors. Baize, S., et al., *Nat Med,* 5(4):423-6 (1999). Although the immunosuppressive mechanisms are yet to be understood, it is probable that the high levels of sGP and the

immunosuppressive peptide in the GP are to blame for the absence of humoral and cellular immune responses in Ebola virus-infected patients.

The proteins that are responsible for the initial inflection of Ebola virus are the viral glycoproteins. Therefore, they are the target for neutralizing

5 antibodies. However, Ebola virus has evolved "tricks" to prevent or delay the host immune response until it is too late to recover from the infection. Conventional approaches in producing vaccines against Ebola virus are likely to be ineffective for the following reasons: (1) viral glycoproteins produced in bacteria, yeast or insect cells are not properly glycosylated and therefore do

10 not have the true antigenicity of the viral proteins; (2) Ebola virus is too dangerous to be produced in large amounts as an inactivated-virus vaccine; and (3) procedures of inactivating the virus often destroy the conformation of the proteins, and therefore alter their antigenicity.

A preferred embodiment of the present invention is a recombinant viral

15 vaccine having nucleic acids encoding one or more antigens of Ebola virus. Restriction maps and full sequence information of the Ebola virus, including the Zaire strain, is available through GenBank.

The genetic vaccine is a recombinant benign virus which is replication defective or incompetent and therefore is incapable of spreading beyond

20 initially infected cells. For example, a recombinant adenoviral vaccine of the present invention mediates high levels of Ebola viral antigen expression for a period of two or more weeks, even though Ebola viral proteins have no functional relevance to recombinant virus function.

In another embodiment of the invention, the recombinant virus

25 expresses one or more modified Ebola virus antigens. The modified Ebola virus antigens are preferably Ebola virus envelope glycoproteins and/or immunogically active parts thereof. Preferably the glycoproteins are modified GP and sGP glycoproteins. The Ebola virus GP and sGP glycoproteins are modified to destroy their pathogenic and immunosuppressive functions, but

30 retain most of their natural antigenicity, since they are expressed, folded, glycosylated, and targeted to the cellular membrane inside the cells that can be productively infected by the Ebola virus. The modifications are carried out

using standard molecular genetic manipulation techniques such as restriction digests and polymerase chain reaction.

A preferred modification of the Ebola virus envelope glycoprotein destroys the infective function of the Ebola virus GP. Any modification that
5   destroys the infective function of Ebola virus can be used, but preferably the modification is a five amino acid deletion in the cleavage site of the GP. *See* Example 1. This cleavage site is composed of five basic amino acid residues, RRTRR, at position 501 from the start of the open reading frame. This deletion may be introduced into the Ebola virus GP cDNA using PCR
10   amplification, which is performed by methods well known in the art.

Another preferred modification of the Ebola virus viral genome prevents synthesis of the sGP. Any modification that prevents synthesis may be employed. Preferably the modification is directed to altering the RNA editing site from UUUUUUU to UUCUUCUU. *See* example 1.
15   Another preferred modification to Ebola virus antigen used in the present vaccines is immunosuppressive (IS) peptide located in GP2. The IS peptide motif is located at amino acids 585-609. A ten amino acid deletion between amino acide 590-600 removes its function. Second, each half of the IS peptide motif is reversed and duplicated. *See* **Figure 2**. This further
20   ensures that its function has been destroyed and also increases its antigenicity.

Further it is readily apparent to those skilled in the art that variations or derivatives of the nucleotide sequences encoding Ebola virus antigen(s) of the present invention can be produced, which alter the amino acid sequence
25   of the encoded protein. The altered expressed antigen(s) may have an altered amino acid sequence, yet still elicit immuneresponses that react with Ebola virus antigen(s), and are considered functional equivalents. In addition, fragments of the full-length genes that encode portions of the full-length protein may also be constructed. These fragments may encode a protein or
30   peptide which elicits antibodies which react with Ebola virus antigen(s), and are considered functional equivalents.

Vaccination of an individual with the vaccines of the present invention results in entrance of adenoviral particles into cells and expression of Ebola

virus antigen(s), such as the envelope glycoproteins, and the immune-stimulating cytokines.  The expression of Ebola virus antigen(s) in cells induces strong and persistent immune responses as if an infection has occurred.  The genetic vaccine has all of the immunogenicity of a natural

5   infection, including expression of the natural viral proteins and long-lasting antigen stimulation, but does not have the pathogenicity of a true viral infection.  In the vaccines of the present invention, the immunosuppressive mechanisms of Ebola virus are disabled, the antigens occur in their natural forms and are associated with the cell membrane, and immune stimulation

10   lasts for weeks.  The effects of this novel vaccine are long lasting and provide high rates of protection against Ebola virus infection.

The present invention is also directed to a method of immunizing a human against Ebola virus infection comprising administering the vaccines described above.  The techniques for administering these vaccines to

15   humans are known to those skilled in the health fields.

By using the genetic vaccine of the present invention, individuals may be immunized against Ebola virus.  Since the genetic vaccine can express high levels of antigens and/or a variety of glycoproteins simultaneously, the vaccinated individuals should be immunized against various strains Ebola

20   virus.  Additionally, since the genetic vaccine can express high levels of cytokines to mimic the body's response to natural viral infection, the body's immune response to such a geneticl vaccine against Ebola virus should be strong and long-lasting, thereby achieving a life-long immunity against the Ebola virus.

25

### 5.    Formulation and Routes of Administration

The present invention also relates to a pharmaceutical composition comprising the vaccine(s) described above, and a pharmaceutically acceptable diluent, carrier, or excipient carrier.  Additionally the vaccine may

30   also contain an aqueous medium or a water containing suspension, often mixed with other constituents in order to increase the activity and/or the shelf life.  These constituents may be salt, pH buffers, stabilizers (such as skimmed milk or casein hydrolysate), emulsifiers, and preservatives.

An adjuvant may be included in the pharmaceutical composition to augment the immune response to the viral antigen expressed from the recombinant virus. Examples of the adjuvant include, but are not limited to, muramyl dipeptide, aluminum hydroxide, saponin, polyanions, anamphipatic

5 substances, bacillus Calmette-Guerin (BCG), endotoxin lipopolysaccharides, keyhole limpet hemocyanin (GKLH), interleukin-2 (IL-2), granulocyte-macrophage colony-stimulating factor (GM-CSF) and cytoxan, a chemotherapeutic agent which is believed to reduce tumor-induced suppression when given in low doses.

10 The present invention also provides kits for enhancing the immunity of a host to a pathogen. These kits may include any one ore more vaccines according to the present invention in combination with a composition for delivering the vaccine to a host and/or a device, such as a syringe, for delivering the vaccine to a host.

15 The vaccine according to the invention can be administered in a conventional active immunization scheme: single or repeated administration in a manner compatible with the dosage formulation, and in such amount as will be prophylactively effective, i.e. the amount of immunizing antigen or recombinant microorganism capable of expressing the antigen that will induce

20 immunity in humans against challenge by the pathogenic virus or bacteria, such virulent Ebola virus, HIV, hepatitis A, B, C, D, and E virus, and bacillus tuberculous. Immunity is defined as the induction of a significant level of protection after vaccination compared to an unvaccinated human.

The vaccine of the present invention, i.e. the recombinant virus, may

25 be administered to a host, preferably a human subject, via any pharmaceutically acceptable routes of administration. The routes of administration include, but are not limited to, intramuscular, intratracheal, subcutaneous, intranasal, intradermal, rectal, oral and parental route of administration. Routes of administration may be combined, if desired, or

30 adjusted depending upon the type of the pathogenic virus to be immunized against and the desired body site of protection.

Doses or effective amounts of the recombinant virus may depend on factors such as the condition, the selected viral or bacterial antigen, the age,

weight and health of the host, and may vary among hosts. The appropriate titer of the recombinant virus of the present invention to be administered to an individual is the titer that can modulate an immune response against the viral or bacterial antigen and elicits antibodies against the pathogenic virus or

5    bacteria from which the antigen is derived. An effective titer can be determined using an assay for determining the activity of immunoeffector cells following administration of the vaccine to the individual or by monitoring the effectiveness of the therapy using well known *in vivo* diagnostic assays. For example, a prophylactically effective amount or dose of a recombinant

10    adenovirus of the present invention may be in the range of from about 100 μl to about 10 ml of saline solution containing concentrations of from about $1 \times 10^4$ to $1 \times 10^8$ plaque forming units (pfu) virus/ml.

One skilled in the art understands that the amount of virus particles to be administered depends, for example, on the number of times the vaccine is

15    administered and the level of response desired.


**6.    Methods of Enhancing the Immunity of a Host to Pathogens**

The present invention also provides methods for enhancing the immunity of a host host to pathogens with the recombinant viruses described

20    above.

In one embodiment, the method is provided for enhancing the immunity of a host to a pathogenic virus. The method comprises: administering to the host a recombinant virus in an amount effective to induce an immune response. The recombinant virus comprises: an antigen

25    sequence heterologous to the benign virus and encoding a viral antigen from a pathogenic virus, expression of the viral antigen eliciting an immune response directed against the viral antigen and cells expressing the viral antigen in the host upon infection of the host by the recombinant virus; and an immuno-stimulator sequence heterologous to the benign virus that encodes

30    an immuno-stimulator whose expression in the host enhances the immunogenicity of the viral antigen. The recombinant virus may preferably be

replication-incompetent and not cause a malignancy naturally associated with the pathogenic virus in the host.

The recombinant virus may be administered to the host via any pharmaceutically acceptable route of administration. The recombinant virus

5    may be administered to the host via a route of intramuscular, intratracheal, subcutaneous, intranasal, intradermal, rectal, oral and parental administration.

In another embodiment, a method is provided for immunizing a host against a pathogenic virus with multiple antigens that elicit strong and long-

10    lasting immune response to the multiple antigens.  The method comprises: administering to the host a recombinant virus in an amount effective to induce an immune response.  The recombinant virus comprises: a plurality of antigen sequences heterologous to the recombinant virus, each encoding a different viral antigen from one or more pathogenic viruses, expression of the plurality

15    of the antigen sequences eliciting an immune response directed against the viral antigen and cells expressing the viral antigen in the host upon infection of the host by the recombinant virus.  The recombinant virus may preferably be replication-incompetent and not cause malignancy that is naturally associated with the pathogenic virus(es) in the host.

20    Optionally, the recombinant virus may also comprise one or more immuno-stimulator sequences heterologous to the recombinant virus that encodes an immuno-stimulator whose expression in the host enhances the immunogenicity of the viral antigen.

In yet another embodiment, a method is provided for immunizing a

25    host against a pathogenic virus by using multiple genetic vaccines or viruses.  Multiple recombinant viruses may carry different antigens in each recombinant virus.  The multiple recombinant viruses may be administered simultaneously or step-wise to the host.

The method comprises: administering to a host a first and second

30    recombinant viruses in an amount effective to induce an immune response, wherein antibodies are produced.  The first recombinant benign virus comprises: an antigen sequence heterologous to the first recombinant virus that encodes a viral antigen from a pathogenic virus, expression of the viral

antigen eliciting an immune response directed against the viral antigen and cells expressing the viral antigen in the host upon infection of the host by the recombinant virus. The second recombinant virus comprises: an immuno-stimulator sequence heterologous to the second recombinant virus that

5     encodes an immuno-stimulator whose expression in the host enhances the immunogenicity of the viral antigen. The first and second recombinant viruses may preferably be replication-incompetent and not cause malignancy naturally associated with the pathogenic virus in the host.

According to the embodiment, the first and second recombinant virus

10     may be any of a benign virus, such as replication-incompetent adenovirus, adeno-associated virus, SV40 virus, retrovirus, herpes simplex virus and vaccinia virus. Optionally, both the first and second recombinant viruses may be replication-incompetent adenovirus. Also optionally, one of the first and second recombinant viruses may be recombinant adenovirus and the other

15     may be recombinant vaccinia virus.

In yet another embodiment, a method is provided for enhancing the immunity of a host to a pathogen. The method comprises: administering to the host a recombinant virus and one or more immuno-stimulators. The recombinant virus may be any of the recombinant viruses described above.

20     In particular, the recombinant virus comprises one or more antigen sequences heterologous to the recombinant virus that encode one or more antigens from the pathogen. Expression of the antigen elicits an immune response directed against the antigen and cells expressing the antigen in the host upon infection of the host by the recombinant virus. The recombinant

25     virus is preferably replication-incompetent and does not cause a malignancy naturally associated with the pathogen in the host. The pathogen may be a pathogenic virus such as HIV, hepatitis virus and Ebola virus, a pathogenic bacteria or parasite.

According to this embodiment, the immuno-stimulator may be any

30     molecule that enhances the immunogenicity of the antigen expressed by the cell infected by the recombinant virus. Preferably, the immuno-stimulator is a cytokine, including, but not limited to interleukin-2, interleukin-8, interleukin-12, $\beta$- interferon, $\lambda$-interferon, $\gamma$-interferon, granulocyte colony stimulating

factor, granulocyte-macrophage colony stimulating factor, and combinations thereof.

The cytokine may be administered into the host in a form of purified protein. Alternatively, the cytokine may be administered in a form of

5      expression vector that expresses the coding sequence of the cytokine upon transfecting or transducing the cells of the host.

Standard procedures for endonuclease digestion, ligation and electrophoresis are carried out in accordance with the manufacturer's or supplier's instructions. Standard techniques are not described in detail and

10     will be well understood by persons skilled in the art.

Practicing the present invention employs, unless otherwise indicated, conventional methods of virology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See e.g. Sambrook, et al. *Molecular Cloning:*

15     *A laboratory Manual; DNA Cloning: A Practical Approach*, vol I & II (D. Glover ed.); *Oligonucleotide Synthesis* (N. Giat, ed.); *Nucleic Acid Hybridization* (B. Hames & S. Higgins, eds., Current Edition); *Transcription and Translation* (B. Hames & S. Higgins, eds., Current Edition); *Fundamental Virology*, 2nd Edition, vol. I & II (B. N. Fields and D. M. Knipe, eds.)

20     The following examples are provided to illustrate the present invention without, however limiting the same thereto.

## EXAMPLES

The following procedures are described to illustrate how to make a genetic vaccine of the present invention against various pathogenic viruses.

5    The genetic vaccine is based on an adenoviral vector with modified antigens derived from the pathogenic virus (e.g., Ebola virus, Hepatitis B virus and HIV) inserted into the adenoviral backbone. Additionally, the recombinant adenovirus also carries multiple genes encoding various cytokines. The recombinant adenovirus is replication-incompetent but still retains adenoviral

10   infectivity.

It is noted that genetic vaccine against other pathogenic viruses, bacteria and parasites may be constructed by one with ordinary skill in the art following similar procedures described in details below.

15

### 1.    Genetic Vaccine against Ebola Virus

Embodiments of the genetic vaccine against Ebola virus and methods of their construction are described in detail as follows.

20   1)    <u>Genetic modification of the Ebola virus membrane glycoproteins</u>

The modifications are carried out using standard molecular genetic manipulation techniques, such as restriction enzyme digests and polymerase chain reaction (PCR).

25   The glycoproteins of Ebola virus are modified to produce the optimal antigen for Ebola virus vaccine. Two modified forms of the GP proteins are constructed to have inactivated immunosuppressive and infectious mechanisms, but retain full natural antigenicity of the wild-type glycoproteins. The mRNA editing signal is deleted to prevent the production of the secreted

30   glycoprotein (sGP), which is immunosuppressive; and (2) the proteolytic cleavage site of the glycoprotein precursor is deleted to prevent the formation of the functional glycoproteins (GP1 and GP2). Sanchez, A., et al., *Proc Natl Acad Sci U.S.A.* 93(8):3602-7 (1996). In one form the immunosuppressive

peptide region is deleted to prevent its function, and in the other form, the immunosuppressive peptide motif is split in order to destroy its function, but retain its immunogenicity. These steps produce effective and safe antigens for the vaccine.

5        The envelope glycoproteins (GP) of the Ebola virus are synthesized as a single precursor protein and cleaved into the two subunits (GP1 and GP2) by a cellular enzyme (furin) during transport. Volchkov, V.E., et al., *Proc Natl Acad Sci U.S.A.*, 95(10):5762-7 (1998). This proteolytic cleavage is essential for the formation of the mature glycoproteins and the release of the fusion

10    peptide located at the C-Terminus of the cleavage site. The mature glycoproteins are incorporated into virions as trimers (each monomer is a heterodimer of GP1 and GP2 linked by a disulfide bond). Sanchez, A., et al., *J. Virol* 72(8):6442-7 (1998). The glycoproteins of Ebola virus are the major proteins exposed on the viral membrane surface, and are responsible for

15    initiating virus entry into host cells. Therefore, they are a primary target for neutralizing antibodies.

       The glycoprotein cleavage site is composed of five basic amino acid residues (RRTRR [SEQ ID NO: 10]) at position 501 from the start site of the open reading frame. The Ebola virus glycoprotein cleavage site is similar to

20    the conserved sequences found in glycoproteins of other viruses, such as in the envelope protein of RSV or MuLV. We have previously shown that deletions or point mutations at these basic amino acid residues can block cleavage and render the glycoproteins non-functional in RSV. Dong, J.Y, et al., *J. Virol* 66(2):865-74 (1992).

25        To destroy the infective functions of the Ebola virus glycoprotein, the five basic amino acid residues in the cleavage site are deleted. This deletion is introduced into the Ebola virus GP cDNA using PCR amplification. Alternatively, the cleavage site can be altered, such as by site specific mutation resulting in elimination of cleavage.

30        Another important feature of the Ebola virus is that two forms of glycoproteins are synthesized from a single gene, a secreted from (sGP) and a membrane-bound form (GP). The two forms are generated as a result of an alternative RNA editing event at a sequence of seven uridines (at location

1020-1028 from the start site), which is highly conserved among all four Ebola virus subtypes. Sanchez, A., et al., *Proc Natl Acad Sci U.S.A.* 93(8):3602-7 (1996). The sGP is synthesized from un-edited mRNA and likely has immunosuppressive functions. The GP is synthesized from an edited mRNA

5    and likely has immunosuppressive functions. The GP is synthesized from an edited mRNA with insertion in one of the seven uridines. This RNA editing causes a frame-shift and results in a translation of the second reading frame that encodes the complete transmembrane glycoprotein (GP2).

To prevent the synthesis of sGP, the RNA editing site is modified from

10    UUUUUUU [SEQ ID NO: 2] to UUCUUCUU [SEQ ID NO: 3] . In the cDNA, the equivalent sequence is AAAAAAA [SEQ ID NO: 4] and AAGAAGAA [SEQ ID NO: 5], respectively. This modification accomplishes two things: (1) all mRNAs encode only the GP (equivalent to the edited form with -1 frame shift); and (2) UUUUUU [SEQ ID NO: 6] encodes the same animo acid

15    residues as UUCUUC [SEQ ID NO: 7], but prevents the possibility of further polymerase slipping at the stretch of the six uridines. The additional editing would cause deletion of one more uridine and further (-2) frame shifting. The mechanism of this modification is diagramed in **Figure 2**.

A third modification may be introduced into the Ebola virus glycoprotein

20    relating to a deletion of the immunosuppressive (IS) peptide located in GP2. The IS peptide motif (amino acid 585-609, form the start site) is highly conserved in filoviruses and has a high degree of homologywith a motif in the glycoproteins of oncogenic retroviruses that has been shown to be immunosuppressive. Volchkov, V.E., et al., *FEBS Lett* 305(3):181-4 (1992);

25    Will, C., et al., *J. Virol* 67(3):1203-10 (1993); Mitani, M., et al., *Proc Natl Acad Sci U.S.A.* 84(1):237-40 (1987); Gatot, J.S., et al., *J. Biol Chem* 273(21):12870-80 (1998); Denner, J., et al., *J Acquir Immune Defic Syndr Hum Retrovirol* 12(5):442-50 (1996). First, a ten amino acid deletion is introduced in the core region of the motif (between amino acid 590-600) to

30    remove its function. Second, each half of the motif is reversed and duplicated to destroy function and increase antigenicity. It is believed that antibodies against the IS peptide may inhibit the immunosuppressive function of the

Ebola viruses during an infection. The basic strategy of this modification is diagrammed in **Figures 3A-3C**.

As illustrated in **Figures 3A-3C**, modification of the immunosuppressive peptide (IS) is made on the GP2 gene. **Figure 3A**

5 illustrates the wild type GP. **Figure 3B** illustrates GP with the 10 amino acid deletion of the IS peptide. **Figure 3C** illustrates the IS peptide, which is split, reversed and duplicated.

With these modifications, Ebola virus glycoproteins are generated that are non-functional, not immunosuppressive, yet they retain the natural

10 antigenicity of GP. These modified GP sequences are used to generate antigens in the vaccines of the present invention against Ebola virus.

DNA sequences of the resulting altered GP genes are confirmed by sequence analysis. The modified GP sequences are then cloned intoplasmid vectors containing DNA elements necessary for efficient expression of these

15 GPs in hostian cells. Expression and correct localization to the cellular membrane is determined by transient transfections of HeLa or 293 cells and analyzed by Western blot and FACS, using polyclonal antibodies from hyperimmunized equine serum and anti-horse secondary antibodies labeled with horse radish peroxidase (HRP) or fluorescent tags, respectively.

20

2) Construction of a series of replication-defective adenoviral vaccines that mediate high levels of expression of the modified Ebola virus GPs

The vaccines of the present invention utilize a recombinant benign

25 virus to carry modified antigens of Ebola virus to trick the host into mounting a robust immune defense against the Ebola virus. The preferred benign virus is a replication-defective adenovirus. These vectors are an excellent choice for vaccine expression, for several reasons. First, adenoviral vectors direct high levels of antigen expression that provides strong stimulation of the immune

30 system. Second, the antigen that they express is processed and displayed in the transduced cells in a way that mimics pathogen-infected cells. This phase is believed to be very important in inducing cellular immunity against infected

cells, and is completely lacking when conventional vaccination approaches
are used. Third, adenoviral vectors infect dendritic cells which are very potent
antigen-presenting cells. Diao, J. et al., *Gene Ther* 6(5):845-53 (1999);
Zhong, L., et al., *Eur J Immunol* 29(3):964-72 (1999); Wan, Y., et al., *Int J*
5    *Oncol* 14(4):771-6 (1999); Wan, Y., et al., *Hum Gene Ther* 8(11):1355-63
(1997). Fourth, these vectors can be engineered to carry immunoenhancing
cytokine genes to further boost immunity. Fifth, adenoviruses naturally infect
airway and gut epithelial cells in humans, and therefore the vaccine may be
delivered through nasal spray or oral ingestion. And finally, the adenoviral
10   vectors of this invention are safe because they are replication-defective
andhave been used in high doses ($10^9$ to $10^{12}$ i.p./dose) in clinical trials for
gene therapy studies. Gahery-Segard, H., et al., *J. Clin Invest* 100(9):2218-
26 (1997); Bellon, G.,et al., *Hum Gene Ther* 8(1):15-25 (1997);
Boucher, R.C., et al., *Hum Gene Ther* 5(5):615-39 (1994). Indeed, even live
15   viruses have been safely used in military recruits to prevent common colds.

This vector-construction system is also used to establish complex
vectors that express multiple genes or regulatory mechanisms. For example,
the vector construct is used to express multiple cytokines along with Ebola
GP antigens in a single complex vector to further enhance the immune
20   induction. Alternatively, antigens and cytokines are placed in separate
vectors. This enables the manipulation of different combinations of cytokines
and antigens by co-transduction (infection) with two or three vectors.

Construction of the adenoviral vectors is diagramed in **Figure 4**. The
cDNA encoding a modified GP(s) is cloned into the left-end (E1 region) of the
25   adenovirus genome using a shuttle vector pLAd (**Figure 4A** left side),
resulting in a shuttle vector pLAd/EBO-GP. The pLAd/EBO-GP vector
contains the left end of the adenoviral genome including the left long terminal
repeats L-TR and the adenoviral packaging signal ψ. Genes encoding
cytokines such as IL-2 and IL-4 are inserted into E4 region of the adenovirus
30   vector using the shuttle vector pRAd (**Figure 4A**, right side), resulting in a
shuttle vector pRAdIL2,4. The pRAdIL2,4 contains the right end of the
adenoviral genome including the right long terminal repeats R-TR.

To construct an adenoviral vector carrying the GP gene only, the shuttle vector pLAd/EBO-GP is digested with appropriate restriction enzymes such as Xba I. The fragment containing the GP gene is ligated to an adenoviral backbone and pRAd vector.

5    To construct an adenoviral vector carrying both the GP gene in the E1 region and cytokine genes in the E4 region, both pLAd/EBO-GP and pRAdIL2, 4 are linearized and ligated to the backbone of the adenovirus (**Figure 4B**).

To generate recombinant adenoviral vectors, the ligated vector

10    genome is transfected into 293 cells, in which only the correctly ligated genome with the two adenoviral terminal repeats can replicate and generate infectious viral particles. Human 293 cells (Graham et al., *J. Gen. Virol.*, 36: 59-72 (1977)), available from the ATCC under Accession No.: CRL1573), has adenovirus E1a and E1b genes stably integrated in its genome. The 293

15    cells supplement the essential E1 gene of adenovirus that has been deleted from the vector backbone. The final vector has E1, E3 and partial E4 deleted and can only replicate in 293 cells, but not in target cells. The adenoviral vectors are amplified in 293 cells and purified by ultracentrifugation in cesium chloride gradients. Titers of vectors are determined by serial dilutions and

20    counting of the infectious particle (ip) after infection of 293 cells.

3)    Determination of immune respones to the genetic vaccine

An *in vitro* assay is used to quantitate the amount of neutralizing

25    antibodies developed in response to the vaccine. The assay is based on a retroviral vector system which is based on a Moloney Murine Leukemia virus system. Vectors and packaging cells expressing GAG and POL proteins have been extensively characterized and are commercially available. A packaging vector construct that carries a β-galactosidase gene as a reporter

30    is used. A novel vector construct expressing the membrane form of the Ebola virus GP is co-transfected with the β-Gal reporter vector resulting in a GAG-

POL packaging cell line, which generates retroviral vector particles with the Ebola virus GP instead of its original envelope protein.

4)      Determination of which modified GP antigen provides better
5       production of neutralizing antibodies in animal models

The adenoviral vaccine vectors carrying the two GP variants are tested for their ability to induce an immune response to the Ebola virus GP in CD-1 mice (Charles River Laboratories; outbred stock of Swiss mice from
10   Rockefeller Institute). Specifically, the neutralizing antibody titers and cytolytic T-lymphocyte (CTL) activities to the Ebola virus GP antigens induced by the GP variants with and without the IS motif are compared. Three groups of 30 8-week old mice are injected subcutaneously with $10^5$ ip of adenoviral vectors expressing GP variant 1 (with IS peptide deleted), GP variant 2 (with
15   IS peptide split and inverted) and β-Galactosidase (control vector), respectively. Six mice from each group are sacrificed (by $CO_2$ asphyxiation and cervical dislocation) at 1, 2, 4, 8 and 16 weeks post-vaccination, and their blood and spleens are harvested. In addition, 6 mice are mock-vaccinated with saline and sacrificed 2 days later to provide preimmunization controls.
20        From mice injected with the control β-Gal vector, tissue sections from the sites of the vector injection are taken, fixed, and stained with the X-gal solution to determine the number and type of vector-transduced cells at various time-points post-infection. In addition, hemolysin staining isperformed to determine the degree of infiltration of various immune cells (neutrophils,
25   macrophages, monocytes, etc.) at the site of the vector delivery.
        Sera from vaccinated animals is assayed for total GP-binding antibodies using a standard 96-well plate ELISA protocol, as has been described. Van Ginkel, F.W., et al., *Hum Gene Ther* 6(7):895-903 (1995); Van Ginkel, F.W., et al., *J Immunol* 159(2):685-93 (1997). Neutralizing
30   activity of the sera is analyzed by monitoring the infectious activity of the Ebola virus GP-pseudotyped retroviral vector (Wool-Lewis, et al., *J. Virol,* 72(4):3155-60 (1998)) on HeLa cells after the vector has been incubated with

various serum concentrations. Expression of β-galactosidase in infected cell lysates serves as an indicator of the neutralizing activity of the serum (the lower the β-gal activity, the more EBO-β-Gal vectors have been neutralized) and is measured using a very sensitive fluorogenic substrate (Galacto-Light

5 kit J) and a fluorescence plate reader. Anti-GP serum-neutralized infection rates are compared to infection rates in the absence of serum and in the presence of non-GP activated serum.

Cytotoxic lymphocytes (CTLs) are extracted from mouse spleen as previously described. Van Ginkel, F.W., et al., *Hum Gene Ther* 1995;

10 6(7):895-903; Dong, J.Y., et al., *Hum Gene Ther* 1996;7(3):319-31. They are mixed with a constant number of detached LnCaP cells (prostate carcinoma cells of epithelial origin) transduced with an adenoviral vector carrying an unmodified Ebola virus GP protein. Ratios of effector: target cells of 10:1, 3:1, and 1:1 are used. The cells are seeded into 96-well plates, and 24 hour

15 later all unattached cells (which include all of the effector CTLs and dead or dying LnCaP cells) are removed, and the remaining viable (adherent) cells are quantitated by the MTT (3-(4,5-dimethylthiazol-20-yl) 2,5-diphenyl tetrazolium bromide) cleavage assay. This assay has been employed in detecting the lymphocyte cytotoxic activity (Ni, J., et al., *J Clin Lab Anal*

20 1996;10(1):42-52) and compares favorably with the radioactive assays in terms of sensitivity, reliability and speed.

5) <u>Immuno-enhancing functions of multiple cytokines</u>
<u>and their effects on the efficacy of the genetic vaccines</u>

25

To augment the effects of the vaccine, a vector-mediated gene transfer to express the immunoenhancing cytokines, such as IL2, IL4, IL12, INF-γ, and GM-CSF is used. Initially, each cytokine is separately cloned or the cytokines are cloned in various combinations into adenoviral vectors separate from the

30 vectors encoding viral antigens. The immunoenhancing effects of individual cytokines or their combinations are studied by co-infecting with a vector encoding the cytokine and the vector carrying the antigens. The titers of

serum antibodies are compared, as well as the time it takes to reach effective titers in animals inoculated with vaccines in combination with different cytokine-expressing vectors. These experiments allow the determination of whether immunoenhancing cytokines induce higher levels of antibodies,

5    shorten the induction time, and prolong the immunity against the Ebola virus.

After determining the best-performing modified GP variant, the extent that the immune response elicited by it is enhanced by co-delivery to the immunization site of vectors carrying various cytokines is analyzed. Interleukin-2, either by itself or in combination with IL-4 or IL-12, has been

10   demonstrated to strongly enhance the activation and proliferation of cytotoxic T-cells, natural killer (NK) cells and B-cells. Michael, B.N., et al., *Cell Immunol* 1994;158(1);105-15; Bruserud, O., et al.,*Eur J. Haematol* 1992;48(4);221-7; Jacobsen, S.E., et al., *Res Immunol* 1995;146(7-8):506-14; Wolf, S.F., et al., *Res Immunol* 1995;146(7-8);486-9; Tepper, R.I., *Res*

15   *Immunol* 1993;144(8):633-7; O'Garra A., et al., *Res Immunol* 1993;144(8):620-5; Ohe, Y., et al., *Int J Cancer* 1993;53(3):432-7; Delespesse, G., et al., *Res Immunol* 1995;146(7-8):461-6. [36-43]. INF-$\gamma$ stimulates the humoral immune response and increases the permeability of the blood vessel walls at the site of its secretion (Chensue, S.W., et al., *J*

20   *Immunol* 154(11):5969-76 (1995); Szente, B.E., et al., *Biochem Biophys Res Commun* 203(3):1645-54 (1994); Adams, R.B., et al., *J. Immunol* 150(6):2356-63(1993)), while Gm-CSF activates and attracts macrophages and other professional APCs to the site of the infection. Bober, L.A., et al., *Immunopharmacology* 29(2):111-9 (1995); Dale, D.C., et al., *Am J. Hematol*

25   57(1):7-15 (1998); Zhao, Y., et al., *Chung Hua I Hsueh Tsa Chih* 77(10): 32-6 (1997).

Six groups of 30 8-week old mice are injected subcutaneously with a mixture of 5x10$^4$ ip of the selected GP variant vector and 5x10$^4$ ip of one of the following vectors: Ad-$\beta$-Gal, Ad-IL2, Ad-IL2/IL4, Ad-IL2/IL12, Ad-IFN-$\gamma$ and

30   Ad-GM-CSF.

Six mice from each group are sacrificed and analyzed at 1, 2, 4, 8 and 16 weeks as described in Example 4. Analysis of total IgG is peformed using

ELISA, neutralizing activity is assayed as interference with the ability of EBO-GP pseudotyped retroviral vector to infect HeLa cells, and anti-GP CTL activity is performed by mixing spleen-extracted CTLs with target LnCaP cells transduced with Ad-EBO-GP construct as described in Example 4. Levels of

5    various cytokines in the serum are also quantitated by ELISA using available commercial assays. In some cases, these assays can distinguish between human and murine versions of the same cytokine, providing direct information on the expression levels of cytokines delivered using Adenovirus vectors and how they correlate with the development of the immune response.

10           After the individual cytokines are analyzed, those that performed best are tested in combinations. Four groups of 30 8-week old mice are injected subcutaneously with a mixture of $5x10^4$ i.p. of the selected GP variant vector and $5x10^4$ i.p. of up to 3 selected cytokine-expressing vectors (if fewer than 3 cytokine vectors are used, i.p. counts are made up with Ad-$\beta$-Gal vector). Six

15   mice from each group are sacrificed at weeks 1, 2, 4, 8 and 16, and analyzed as described above.

             To verify the robust and reproducible nature of the immune response to the GP vector and multiple cytokines in different species, the experiment as described above is reproduced in rabbits. Five groups of six white New

20   Zealand rabbits are injected into the thigh muscle with one of the following vector combinations: the Ad-$\beta$-Gal vector ($10^6$ ip), the selected GP vector (2.5 x $10^5$ ip plus $7.5x10^5$ ip of Ad-$\beta$-Gal), and three cytokine vector combinations ($2.5x10^5$ of each cytokine vector) plus the GP vector ($2.5x10^5$ ip), as described above. The animals are bled 2 days prior to vaccination (pre-

25   immune bleed) and then according to the schedule described above. 5 to 10 ml of blood will be extracted per session. Analysis is performed in a similar fashion to that of mice (see above).

             Because genes coding for human cytokines are used in mouse and rabbit models, it is possible that their immune systems will have a non-

30   identical (to human) response to those proteins. However, a high degree of homology exists between human and mouse cytokines and their receptors, and published reports on experiments using human or other hostian cytokines

in mice indicate a high level of equivalency. If necessary, species-specific versions of these cytokines can be obtained and cloned into the adenoviral vectors of the present invention for species-targeted cytokine activity studies.

5   6)   <u>Optimizing the efficiency and rates of administration of the vaccine and conducting safety and pathogen challenge studies in non-primate and primate animal models</u>

After determining the best combinations of the cytokines and antigens,
10  the final version of the vaccine vectors are constructed. These complex recombinant adenoviral vectors deliver combinations of cytokines and antigens into target cells using a single vector. Dose-titer analysis in mice and rabbits are conducted to identify the lowest dose required to generate maximallevels of immune responses. Different routes of vaccine
15  administration, such as intramuscular and intravenous injection, oral ingestion and nasal sprays are compared. For safety studies, dose escalation experiments in mice and rabbits are conducted until toxicity is observed or until levels ten times the effective dose have been reached. Finally, additional safety and pathogen challenge experiments are conducted in
20  primates.

## 2.   Genetic Vaccine Against HIV

Specific embodiments of the genetic vaccines against HIV and methods of their construction are described in detail as follows.

25

A.   <u>Construction of replication-defective adenoviral vaccines agains HIV</u>

*1)   Ad-E.T.R/IL2*

An adenoviral vector, Ad-E.T.R/IL2, was constructed to carry coding
30  sequences for multiple HIV antigens including Env, Tat, and Rev proteins, and interleukin-2 (IL-2) in the same vector. Expression of the HIV antigens and IL-2 is separately controlled by promoters located in different regions of the adenoviral vector. This design is believed to be able to ensure high level

expression of both the viral antigens and the immuno-stimulator IL-2 and to enhance immunogenicity of the adenoviral vaccine. As shown by experimental data presented in the next section, this adenoviral vector is capable of eliciting strong humoral immune response in animals against HIV

5 antigens.

The adenoviral vector, Ad-E.T.R/IL2, was constructed using strategies similar to those for constructing the adenoviral vaccines against Ebola virus as described in detail above. Briefly, EcoRI/XhoI restriction fragment from HIV-1 strain BH10 (HIV-1 or HTLV-IIIB, clade B, Accession No: M15654),

10 which encodes wildtype envelope gp160 (full length gp 120 and gp41), full length wildtype Tat and full length wild type Rev, was inserted into the left end (E1 region) of the adenoviral genome using a shuttle vector, resulting in a shuttle vector pLAd-E.T.R (**Figure 16A**). DNA sequence of this EcoRI/XhoI restriction fragment [SEQ ID NO: 14] is shown in **Figure 38**.

15 The sequence encoding IL-2 (with a silent mutation CTA to CTT at amino acid position 79 to delete the XbaI site) was inserted into E4 region of the adenoviral genome using a shuttle vector, resulting in a shuttle vector pRAd-ORF6-IL2 (**Figure 16B**). DNA sequence encoding this mutated IL-2 (IL-2ΔX) [SEQ ID NO: 15] is shown in **Figure 39**.

20 Both pLAd-E.T.R and pRAd-OFR6-IL2 were linearized using appropriate restriction enzymes such as Xba I and EcoRI and ligated to the backbone of the adenovirus (**Figure 4B**), resulting in the recombinant adenoviral vector Ad-E.T.R/IL2.

25 2) *Ad-3C/E$^m$ΔCΔT$^{300}$-G*

Another adenoviral vector, Ad-3C/E$^m$ΔCΔT$^{300}$-G, was constructed to carry coding sequences for multiple HIV antigens including a modified Env (gp160) with deletion of the cleavage site between gp120 and gp41 and the cytosolic domain and Gag proteins, and three different cytokines (IL-2 with

30 silent mutation CTA to CTT at amino acid position 79 to delete the XbaI site, INF-γ, and GMCSF) in the same vector. Expression of the HIV antigens and the cytokines is separately controlled by promoters located in different regions

of the adenoviral vector. This design is believed to be able to ensure high level expression of both the viral antigens and the immuno-stimulators and to enhance immunogenicity of the adenoviral vaccine. As shown by experimental data presented in the next section, this adenoviral vector is

5    capable of eliciting strong humoral immune response in animals against HIV antigens.

The adenoviral vector, Ad-3C/E$^m$ΔCΔT$^{300}$-G, was constructed using strategies similar to those for constructing the adenoviral vaccines against Ebola virus as described in detail above. Briefly, the sequence from HIV-1

10   strain BH10 that encodes Env/gp160 (nucleotide position 5580-7850) was modified to delete the sequences encoding the cleavage site (REKR [SEQ ID NO: 11] encoded by nucleotide at position 7101-7112) and the cytosolic domain of 100 amino acids in length (encoded by nucleotide at position 7850-8150), and then, along with the sequence encoding a full length Gag, inserted

15   into the right end (E4 region) of the adenoviral genome using a shuttle vector. DNA sequence of this modified Env (E$^m$ΔCΔT (BH10) [SEQ ID NO: 16] and that of the full length Gag [SEQ ID NO: 17] (amino acid sequence of which is SEQ ID NO: 18, **Figure 41B** ) are shown in **Figures 40** and **41A**, respectively.

20   These two HIV antigens are expressed separately from a CMV promoter via a retroviral splicing donor (SD) and acceptor (SA) mechanism at two splicing acceptor sites, SA$_1$, and SA$_2$. To facilitate efficient cloning of various gene fragments, a cloning vector SD/SA1.2.3 was constructed to include a retroviral SD site and multiple retroviral SA sites, SA$_1$, SA$_2$, SA$_3$ and

25   SA$_4$. In this example, the SD and SA sites were derived from Moloney murine leukemia virus (MMLV) and their sequences are shown below:

SD site (MMLV nt 204-210): AGGTAAG  [SEQ ID NO: 72]; and
SA$_{1-4}$ site (MMLV nt 560-568): CTGCTGCAG  [SEQ ID NO: 73].

Each of the SD site, and the SA$_1$, SA$_2$, SA$_3$ and SA$_4$ (SA$_{1-4}$) sites which share

30   the same sequence was inserted into the multiple cloning site of a cloning vector pSP73 by using standard PCR mutagenesis. As illustrated in **Figure 37**, SA$_1$ was inserted immediately downstream from SD site, followed by SA$_2$,

SA$_3$ and SA$_4$. To test the levels of expression of multiple genes via the SD/SA mechanism, the GFP (green fluorescence protein) gene was inserted between SD/SA$_1$ and SA$_2$, SA$_2$ and SA$_3$, SA$_3$ and SA$_4$, and after SA$_4$. The ratio of expression levels in these four sites is 10:1:5:4.

5          DNA sequences encoding E$^m$ΔCΔT and Gag were inserted into the cloning vector SD/SA1.2.3 after SD/SA$_1$, and SA$_2$, respectively. The resulting vector was digested with EcoRV and XhoI and the fragment containing E$^m$ΔCΔT and Gag was inserted into an adenoviral shuttle vector, resulting in pRAd-ORF6-cmv- E$^m$ΔCΔT$^{300}$-G (**Figure 17A**). Shuttle vectors capable of
10     expressing other proteins (as shown below) via the retroviral SD/SA mechanism were constructed using the same strategy.

          Sequences encoding multiple immuno-stimulators, including IL-2 (with a silent mutation caused by deletion of Xba I site), INF-γ, and GMCSF, were inserted into E1 region of the adenoviral genome using a shuttle vector.
15     These three immuno-stimulators are expressed separately from another CMV promoter via a retroviral splicing donor (SD) and acceptor (SA) mechanism at three splicing acceptor sites, SA$_1$, SA$_2$, and SA$_3$. The shuttle vector produced is designated pLAd-3C (**Figure 17B**).

          Both pRAd-ORF6-cmv- E$^m$ΔCΔT$^{300}$-G and pLAd-3C were linearized
20     using appropriate restriction enzymes such as Xba I and EcoRI and ligated to the backbone of the adenovirus (**Figure 4B**), resulting in the recombinant adenoviral vector Ad-3C/E$^m$ΔCΔT$^{300}$-G.


     *3)     Ad-3C/E$^m$ΔCΔT$^{99}$.T.R-G*

25          Yet another adenoviral vector, Ad-3C/E$^m$ΔCΔT$^{99}$.T.R-G, was constructed to carry coding sequences for multiple HIV antigens from strain pNL4-3 (Accession No: M19921) including a modified Env (gp160 with deletion of the cleavage site and the cytoplasmic domain of 33 amino acids in length), full length Rev and Gag proteins, and three different cytokines (IL-2
30     with silent mutation CTA to CTT at amino acid position 79 to delete the XbaI site, INF-γ, and GMCSF) in the same vector. Expression of the HIV antigens and the cytokines is separately controlled by promoters located in different

regions of the adenoviral vector. This design is believed to be able to ensure high level expression of both the viral antigens and the immuno-stimulators and to enhance immunogenicity of the adenoviral vaccine. As shown by experimental data presented in the next section, this adenoviral vector is

5      capable of eliciting strong humoral immune response in animals against HIV antigens.

The adenoviral vector, Ad-3C/$E^m\Delta C\Delta T^{99}$.T.R-G, was constructed using strategies similar to those for constructing the adenoviral vaccines against Ebola virus as described in detail above. Briefly, the sequence from HIV-1

10     strain pNL4-3 that encodes Env/gp160 (nucleotide position 6221-8686) was modified to delete the sequences encoding the cleavage site (encoded by nucleotide at position 7736-7747) and the cytosolic domain (encoded by nucleotide at position 8687-8785) in length, and then, along with sequences encoding full length Tat, Rev, and Gag (from HIV strain BH10), inserted into

15     the right end (E4 region) of the adenoviral genome using a shuttle vector.

These three HIV antigens are expressed separately from a CMV promoter via a retroviral splicing donor (SD) and acceptor (SA) mechanism at three splicing acceptor sites, SA, $SA_2$, and $SA_3$. The shuttle vector produced is designated pRAd-$E^m\Delta C\Delta T^{99}$.T.R-G (**Figure 18**). DNA sequence encoding

20     the modified Env, and full length Tat and Rev [SEQ ID NO: 19] is shown in **Figure 42**. DNA and amino acid sequences of the full length Gag from HIV strain BH10 [SEQ ID NO: 17] are shown in **Figures 41A** and **41B**, respectively.

The shuttle vectors, pRAd-$E^m\Delta C\Delta T^{99}$.T.R.-G and pLAd-3C (**Figure**

25     **17B**) were linearized using appropriate restriction enzymes such as Xba I and EcoRI and ligated to the backbone of the adenovirus (**Figure 4B**), resulting in the recombinant adenoviral vector Ad-3C/$E^m\Delta C\Delta T^{99}$.T.R.-G.


*4)      Ad-$E^m\Delta V_{1,2} \Delta C \Delta T^{99}$.T.R-IL2/G.IL2*

30     Yet another adenoviral vector, Ad-$E^m\Delta V_{1,2} \Delta C \Delta T^{99}$.T.R/G.IL2, was constructed to carry coding sequences for multiple HIV antigens from HIV-1 strain pNL4-3. The sequence from HIV-1 strain pNL4-3 that encodes

Env/gp160 (nucleotide position 6221-8686) was modified to delete the sequences encoding the V1 and V2 loops at position 6602-6796 nt and insert nucleotide sequence GGA GCT GGT [SEQ ID NO: 12] that encodes amino acid sequence GAG [SEQ ID NO: 13]. This HIV Env/gp160 was also

5   modified to delete the cleavage site encoded by nucleotide at position 7736-7747 ($\Delta$C) and the 33-aa cytosolic domain encoded by nucleotide at position 8687-8785 ($\Delta T^{99}$). Along with the sequences encoding full length Rev (R) and Tat (T), the modified *env* was inserted into the left end (E1 region) of the adenoviral genome using a shuttle vector. DNA sequence encoding the insert

10   ($E^m\Delta V_{1,2}$ $\Delta$C $\Delta$T.T.R) [SEQ ID NO: 20] is shown in **Figure 43**.

Additionally, IL-2 (with a silent mutation caused by deletion of Xba I site, DNA SEQ ID NO: 15) was inserted downstream from the modified *env*. Both the modified Env and IL-2 are expressed separately from a CMV promoter via a retroviral splicing donor (SD) and acceptor (SA) mechanism at

15   two splicing acceptor sites, $SA_1$ and $SA_2/SA_3$. The shuttle vector produced is designated pLAd- $E^m\Delta V_{1,2}$ $\Delta$C $\Delta$T.T.R-IL2 (**Figure 19A**).

Sequences encoding IL-2 (with a silent mutation caused by deletion of Xba I site, DNA SEQ ID NO: 15) and Gag from HIV-1 strain BH10 (nt 112-1650, DNA SEQ ID NO: 17) were inserted into E4 region of the adenoviral

20   genome using a shuttle vector. These two proteins are expressed separately from a CMV promoter via a retroviral splicing donor (SD) and acceptor (SA) mechanism at two splicing acceptor sites, $SA_1$ and $SA_2$. The shuttle vector produced is designated pRAd-ORF6-G.IL2 (**Figure 19B**).

Both pLAd-cmv- $E^m\Delta V_{1,2}$ $\Delta$C $\Delta$T.T.R-G and pRAd-ORF6-G.IL2 were

25   linearized using appropriate restriction enzymes and ligated to the backbone of the adenovirus (**Figure 4B**), resulting in the recombinant adenoviral vector Ad-$E^m\Delta V_{1,2}$ $\Delta$C $\Delta$T.T.R-G/G.IL2.

### 5)   *Ad-$E^m\Delta$C.T.R.N/G.IL2*

30   Yet another adenoviral vector, Ad-$E^m\Delta$C.T.R.N/G.IL2, was constructed to carry coding sequences for multiple HIV antigens from HIV-1 strain BH10. The sequence from HIV-1 strain BH10 that encodes full length Env/gp160

(nucleotide position 5580-8150), Tat, Rev, and Nef was modified by deleting the sequence encoding the cleavage site of Env and inserting a SpeI restriction site. DNA sequence of this insert [SEQ ID NO: 21] is shown in **Figure 44**, and was inserted into the left end (E1 region) of the adenoviral

5   genome using a shuttle vector, resulting in a shuttle vector pLAd-E$^m$ΔC.T.R.N **(Figure 20)**.

Both pLAd-E$^m$ΔC.T.R.N and pRAd-ORF6-G.IL2 **(Figure 19B)** were linearized using appropriate restriction enzymes and ligated to the backbone of the adenovirus **(Figure 4B)**, resulting in the recombinant adenoviral vector

10  Ad-E$^m$ΔC.T.R.N/G.IL2.

6)   *Ad-E$^m$ΔC.N /G.IL2*

Yet another adenoviral vector, Ad-E$^m$ΔC.N/G.IL2, was constructed to carry coding sequences for multiple HIV antigens from HIV-1 strain BH10.

15  The sequence from HIV-1 strain BH10 that encodes full length Env/gp160 (nucleotide position 5580-8150, with preceding Kozak sequence), Tat, Rev, and Nef was modified by deleting the sequences encoding the cleavage site of Env, Tat and Rev, and inserting a SpeI restriction site. DNA sequence of this insert [SEQ ID NO: 22] is shown in **Figure 45**, and was inserted into the

20  left end (E1 region) of the adenoviral genome using a shuttle vector, resulting in a shuttle vector pLAd-E$^m$ΔC.N **(Figure 21)**.

Both pLAd-E$^m$ΔC.N and pRAd-ORF6-G.IL2 **(Figure 19B)** were linearized using appropriate restriction enzymes and ligated to the backbone of the adenovirus **(Figure 4B)**, resulting in the recombinant adenoviral vector

25  Ad-E$^m$ΔC.N/G.IL2.

7)   *Ad-E$^m$ΔCΔT$^{300}$.T /G.IL2*

Yet another adenoviral vector, Ad-E$^m$ΔC ΔT$^{300}$.T/G.IL2, was constructed to carry coding sequences for multiple HIV antigens from HIV-1

30  strain BH10. The sequence from HIV-1 strain BH10 that encodes full length Env/gp160 (nucleotide position 5580-8150) was modified by deleting the sequence encoding the cleavage site and a 300 nt sequence encoding the

cytosolic domain, but still including sequence for full length Tat (T). DNA sequence of this insert [SEQ ID NO: 23] is shown in **Figure 46**, and was inserted into the left end (E1 region) of the adenoviral genome using a shuttle vector, resulting in a shuttle vector pLAd-E$^m$ΔC ΔT$^{300}$.T (**Figure 22**).

5    Both pLAd-E$^m$ΔC ΔT$^{300}$.T and pRAd-ORF6-G.IL2 (**Figure 19B**) were linearized using appropriate restriction enzymes and ligated to the backbone of the adenovirus (**Figure 4B**), resulting in the recombinant adenoviral vector Ad-E$^m$ΔC ΔT$^{300}$.T/G.IL2.

10   8)    Ad-E$^m$ΔC/ E$^m$ΔC

Yet another adenoviral vector, Ad-E$^m$ΔC/ E$^m$ΔC, was constructed to carry coding sequences for two copies of a modified Env from HIV-1 strain BH10. The sequence from HIV-1 strain BH10 that encodes full length Env/gp160 (nucleotide position 5580-8150, preceding Kozak sequence) was
15   modified by deleting the sequence encoding the cleavage site. DNA sequence of the modified Env [SEQ ID NO: 24] is shown in **Figure 47**, and was inserted into the left end (E1 region) of the adenoviral genome using a shuttle vector, resulting in a shuttle vector pLAd-E$^m$ΔC (**Figure 23A**).

The DNA sequence encoding the modified Env (E$^m$ΔC) [SEQ ID NO:
20   24] was also inserted into E4 region of the adenoviral genome using a shuttle vector, resulting in shuttle vector pRAd-ORF6-E$^m$ΔC (**Figure 23B**).

Both pLAd-E$^m$ΔC and pRAd-ORF6-E$^m$ΔC were linearized using appropriate restriction enzymes and ligated to the backbone of the adenovirus (**Figure 4B**), resulting in the recombinant adenoviral vector Ad-E$^m$ΔC/ E$^m$ΔC.

25

9)    Ad-E$^m$.V3$^m$/G.IL-2

Yet another adenoviral vector, Ad-E$^m$.V3$^m$/G.IL-2, was constructed to carry coding sequences for modified HIV-1 Env having multi-clade V3 loops and Gag, and IL-2. Sequences encoding V3 loop from clade B, A, C, D, E, F,
30   and G within Group M of HIV-1 are shown in **Figure 48**. As shown in **Figure 48**, for clade B (HIV-1 strain BH10) DNA sequence encoding V3 loop, nt 885-992 [SEQ ID NO: 25], was chosen. In this particular embodiment, for clade A

(HIV-1 strain 192UG037WHO.01083hED) DNA sequence encoding V3 loop, nt 888-992 [SEQ ID NO: 26], was chosen. For clade C (HIV-1 strain 192BR025WHO.01093hED) DNA sequence encoding V3 loop, nt 876-980 [SEQ ID NO: 27], was chosen. For clade D (HIV-1 strain 192UG024.2) DNA

5  sequence encoding V3 loop, nt 888-989 [SEQ ID NO: 28], was chosen. For clade E (HIV-1 strain 193TH976.17) DNA sequence encoding V3 loop, nt 894-998 [SEQ ID NO: 29], was chosen. For clade F (HIV-1 strain 193BR020.17) DNA sequence encoding V3 loop, nt 888-992 [SEQ ID NO: 30], was chosen. For clade G (HIV-1 strain 192RU131.9) DNA sequence

10  encoding V3 loop, nt 885-989 [SEQ ID NO: 31], was chosen.

The DNA sequences encoding V3 loops from HIV clade A, C, D, E, F, and G were ligated by PCR to form a single fragment containing multiclade V3 loops. Primers for cloning these V3 loops from their cognate HIV clades are listed in **Figure 57**. Since V3 loop of HIV clade B is already contained in

15  the backbone of HIV-1 gp120, the cloned V3 loops from clade A, C, D, E, F, and G were inserted after V3 loop of clade B.

**Figure 24** illustrate a process for generating the ligated multiclade V3 loops by PCR and subsequent cloning into a construct encoding a modified gp120 of clade B. As illustrated in **Figure 24**, each of the gene fragments

20  encoding the envelope V3 loop region from clade A, C, D, E, F, and G was individually amplified by PCR using a set of forward and reverse primers listed in **Figure 57**. Parameters for the PCR cycles are the following:

denature:     94 $^0$C for 1 min;
annealing:    50 to 60 $^0$C for 30 sec; and
25  extension:    72 $^0$C for 1 min;
for 20 cycles.

The PCR product encoding V3 loop of one clade was ligated with another using PCR. For example, the PCR products encoding V3 loops of clade A and C were mixed together, ligated and amplified by PCR using the

30  primers 1 and 4 as shown in **Figure 24**, procucing an A/C fragment. Similarly, a PCR product encoding the ligated V3 loops of clade D and E was

generated using primers 5 and 8, producing a D/E fragment; and clade F and G using primers 9 and 12 (**Figure 24**), producing a F/G fragment.

Still referring to **Figure 24**, the A/C and D/E fragments were ligated by PCR using primers 1 and 8 and cloned into a vector at EcoRI and BamHI

5  sites. The F/G fragment was restriction digested with BamH1 and XbaI and fused with the sequence A/C/D/E to generate the multi-clade sequence ACDEFG (V3$^m$).

To generate two repeats of the multi-clade ACDEFG sequence, the final PCR product encoding the multi-clade ACDEFG sequence was

10  restriction digested with AvaI (at primer 1 and 12) and re-ligated head-to-tail, yielding the two repeat multiclade sequence 2x V3$^m$. The DNA sequence encoding V3$^m$ or 2x V3$^m$ was then inserted after the sequence encoding V3 loop of clade B in a construct encoding gp120 which was modified as follows.

DNA sequence encoding Env (nt 5580-8150) from HIV strain BH10

15  (clade B) was modified by a) deleting the sequence encoding the cleavage site (nt 7101-7112); b) deleting V1 and V2 loops (nt 5961-6161) and inserting nucleotide sequence GGA GCT GGT [SEQ ID NO: 12] that encodes amino acid sequence GAG [SEQ ID NO: 13]; c) inserting the multi-clade V3 loop (V3$^m$) sequence at position nt 6572; and d) replacing gp41 transmembrane

20  domain sequence with a GPI anchor sequence encoding glycophosphatidyl inositol, SWLLLLLLSLSLLQATDFMSL [SEQ ID NO: 9]. DNA sequence encoding this modified Env [SEQ ID NO: 32] (the amino acid sequence of which is SEQ ID NO: 33, **Figure 49B**) is shown in **Figure 49A**, and was inserted into the left end (E1 region) of the adenoviral genome using a shuttle

25  vector, resulting in a shuttle vector pLAd-E$^m$.V3$^m$ (**Figure 25**).

Both pLAd-E$^m$.V3$^m$ and pRAd-ORF6-G.IL2 (**Figure 19B**) were linearized using appropriate restriction enzymes and ligated to the backbone of the adenovirus (**Figure 4B**), resulting in the recombinant adenoviral vector Ad-E$^m$.V3$^m$/G.IL2.

30

*10)    Shuttle Vector pLAd-E$^m$.2xV3$^m$*

To increase the expression level of the multi-clade V3 loops, the sequnece encoding two repeats of V3$^m$ sequence (2xV3$^m$, constructed above) was inserted into the sequence encoding the modified Env described in section 9) above. The resulting shuttle vector is designated pLAd-E$^m$.2xV3$^m$

5    and is shown in **Figure 26**.

*11)    Shuttle Vectors encoding p17 and/or p24*

In nature the Pr55 Gag protein can be processed into four different proteins, p17MA, p24CA, p7NC, and p6. The p17MA protein remains

10    associated with the inner side of the lipid envelope, and plays an important role in anchoring of envelope to the viral particle. The p24CA protein of all retroviruses contains a major homology region (MHR) that is required for efficient viral replication and particle production. Elispot data obtained implicates that p17MA (or p17) and p24CA (or p24) may have contributed

15    significantly the specific CTL response in the Pr55 gag protein in peptide mapping experiments. According to the present invention, these HIV structural proteins are expressed by the recombinant virus to elicit specific CTL response to HIV infection. Further, these structure proteins can be modified to include a signal peptide (e.g., the HIV gp120 signal peptide

20    encoded by SEQ ID NO: 74: atgagagtgaaggagaaatatcagcacttgtggagatggggggtggagatggggcaccatgctccttggga tgttgatgatctgtagtgct) sequence which facilitates the secretion of these intracellular proteins by the infected cells. Moreover, by adding a membrane anchoring domain (e.g, the HIV gp41 transmembrane domain encoded by

25    SEQ ID NO: 75: ttattcataatgatagtaggaggcttggtaggtttaagaatagtttttgctgtactttctgtagtgaatagagttagg cagggatattcaccattatcgtttcagacccacctcccaatcccgagggga) to the secreted form of the HIV protein, such a modified HIV structural protein is rendered membrane bound, which better presents the HIV antigen to the body's

30    immune system. These modifications may confer a much stronger immunogenicity to the mutant antigen than the native antigen which are trapped intracellularly.

Adenoviral shuttle vectors were constructed to encode the processed Gag proteins, p17, p24, and p17/24, each in three different forms: natural form, secreted form and membrane bound form.

DNA sequences of p17/p24 in the three forms [SEQ ID NOs: 34-36]
5    are shown in **Figure 50A** (corresponding amino acid sequences [SEQ ID NOs: 37-39], **Figure 50B**) and were each inserted into E4 region of the adenoviral genome using a shuttle vector, resulting in shuttle vector pRAd-ORF6-p17/24 (natural form, **Figure 27A**), pRAd-ORF6-p17/24sec (secreted form, **Figure 27B**), and pRAd-ORF6-p17/24MB (membrane-bound form,
10   **Figure 27C**), respectively.

DNA sequences of p17 in the three forms [SEQ ID NOs: 40-42] are shown in **Figure 51A** (corresponding amino acid sequences [SEQ ID NOs: 43-45], **Figure 51B**) and were each inserted into E4 region of the adenoviral genome using a shuttle vector, resulting in shuttle vector pRAd-ORF6-p17
15   (natural form, **Figure 28A**), pRAd-ORF6-p17sec (secreted form, **Figure 28B**), and pRAd-ORF6-p17MB (membrane-bound form, **Figure 28C**), respectively.

DNA sequences of p24 in the three forms [SEQ ID NOs: 46-48] are shown in **Figure 52A** (corresponding amino acid sequences [SEQ ID NOs: 49-51], **Figure 52B**) and were each inserted into E4 region of the adenoviral
20   genome using a shuttle vector, resulting in shuttle vector pRAd-ORF6-p24 (natural form, **Figure 29A**), pRAd-ORF6-p24sec (secreted form, **Figure 29B**), and pRAd-ORF6-p24MB (membrane-bound form, **Figure 29C**), respectively.

The pLAd- and pRAd-shuttle vectors constructed above can be combined in a combinatorial way to generate a wide variety of recombinant
25   adenoviral vectors. The following are just a few examples of such recombinant adenoviral vectors.

*12)   Ad-E$^m$.2xV3$^m$/p17/24MB*

**Figures 30A-B** illustrate the construction of a recombinant adenoviral
30   vector encoding modified Env containing two copies of multi-clade V3 loops and p17/p24 in membrane-bound form. As illustrated in **Figures 30A-B**, pLAd-E$^m$.2xV3$^m$ (details of the vector shown in **Figure 26**) and pRAd-ORF6-

p17/24MB (details of the vector shown in **Figure 27C**) were linearized using EcoR1 and Xba1 restriction enzymes and ligated to the backbone of the adenovirus, resulting in the recombinant adenoviral vector Ad-$E^m.2xV3^m$/p17/24MB.

5

*13)    Ad-$E^m.2xV3^m$/p17MB*

   **Figures 31A-B** illustrate the construction of a recombinant adenoviral vector encoding modified Env containing two copies of multi-clade V3 loops and p17 in membrane-bound form. As illustrated in **Figures 31A-B**, pLAd-

10    $E^m.2xV3^m$ (details of the vector shown in **Figure 26**) and pRAd-ORF6-p17MB (details of the vector shown in **Figure 28C**) were linearized using EcoR1 and Xba1 restriction enzymes and ligated to the backbone of the adenovirus, resulting in the recombinant adenoviral vector Ad-$E^m.2xV3^m$/p17MB.

15  *14)    Ad-$E^m.2xV3^m$/p24MB*

   **Figures 32A-B** illustrate the construction of a recombinant adenoviral vector encoding modified Env containing two copies of multi-clade V3 loops and p24 in membrane-bound form. As illustrated in **Figures 32A-B**, pLAd-$E^m.2xV3^m$ (details of the vector shown in **Figure 26**) and pRAd-ORF6-p24MB

20    (details of the vector shown in **Figure 29C**) were linearized using EcoR1 and Xba1 restriction enzymes and ligated to the backbone of the adenovirus, resulting in the recombinant adenoviral vector Ad-$E^m.2xV3^m$/p24MB.

   *15)    Ad-$E^m \Delta C \Delta T^{300}.2xV3^m.T$./p17/24sec*

25    DNA sequence encoding Env (including Tat1 (nt 5189-5403) and Tat2 (7734-7779)) from HIV strain BH10 was modified by a) deleting the sequence encoding the cleavage site (nt 7101-7112); b) deleting V1 and V2 loops (nt 5961-6161) and inserting nucleotide sequence GGA GCT GGT [SEQ ID NO: 12] that encodes amino acid sequence GAG [SEQ ID NO: 13]; c) inserting

30    two copies of the multi-clade V3 loop ($2xV3^m$) sequence at position nt 6572; and d) deleting the cytosolic domain of 100 amino acids in length (encoded by nucleotide at position 7850-8150). DNA sequence encoding this modified

Env [SEQ ID NO: 52] (the amino acid sequence of which is SEQ ID NO: 53, **Figure 53B**) is shown in **Figure 53A**, and was inserted into the left end (E1 region) of the adenoviral genome using a shuttle vector, resulting in a shuttle vector pLAd-E$^m$ΔCΔT$^{300}$.2xV3$^m$.T (**Figure 33**).

5  Both pLAd-E$^m$ΔCΔT$^{300}$.2xV3$^m$.T and pRAd-ORF6-p17/24sec (**Figure 27B**) were linearized using appropriate restriction enzymes and ligated to the backbone of the adenovirus (**Figure 4B**), resulting in the recombinant adenoviral vector Ad-E$^m$ΔCΔT$^{300}$.2xV3$^m$.T./p17/24sec.

10  *16)  Ad-E$^m$ΔCΔT$^{300}$.2xV3$^m$.T./p17/24MB*

Both pLAd-E$^m$ΔCΔT$^{300}$.2xV3$^m$.T (**Figure 33**) and pRAd-ORF6-p17/24MB (**Figure 27C**) were linearized using appropriate restriction enzymes and ligated to the backbone of the adenovirus (**Figure 4B**), resulting in the recombinant adenoviral vector Ad-E$^m$ΔCΔT$^{300}$.2xV3$^m$.T./p17/24MB.

15

*17)  Ad-E$^m$ΔCΔT$^{99}$.2xV3$^m$.T.R/p17/24sec*

DNA sequence encoding Env (including Tat1 (nt 5189-5403), Rev1 (nt 5328-5403), Tat2 (7734-7779) and Rev2 (7734-8008)) from HIV strain BH10 was modified by a) deleting the sequence encoding the cleavage site (nt 20  7101-7112); b) deleting V1 and V2 loops (nt 5961-6161) and inserting nucleotide sequence GGA GCT GGT [SEQ ID NO: 12] that encodes amino acid sequence GAG [SEQ ID NO: 13]; c) inserting two copies of the multi-clade V3 loop (2xV3$^m$) sequence at position nt 6572; and d) deleting the cytosolic domain of 33 amino acids in length (nt 8687-8785). DNA sequence 25  encoding this modified Env [SEQ ID NO: 54] (the amino acid sequence of which is SEQ ID NO: 55, **Figure 54B**) is shown in **Figure 54A**, and was inserted into the left end (E1 region) of the adenoviral genome using a shuttle vector, resulting in a shuttle vector pLAd-E$^m$ΔCΔT$^{300}$.2xV3$^m$.T.R (**Figure 34**).

Both pLAd-E$^m$ΔCΔT$^{300}$.2xV3$^m$.T.R and pRAd-ORF6-p17/24sec (**Figure 27B**) were linearized using appropriate restriction enzymes and ligated to the 30  backbone of the adenovirus (**Figure 4B**), resulting in the recombinant adenoviral vector Ad-E$^m$ΔCΔT$^{99}$.2xV3$^m$.T.R/p17/24sec.

*18)   Ad-E^m$\Delta$CAT^{99}.2xV3^m.T.R/p17/24MB*

   Both pLAd-E^m$\Delta$CAT^{99}.2xV3^m.T.R (**Figure 34**) and pRAd-ORF6-
p17/24MB (**Figure 27C**) were linearized using appropriate restriction

5   enzymes and ligated to the backbone of the adenovirus (**Figure 4B**), resulting

   in the recombinant adenoviral vector Ad-E^m$\Delta$CAT^{99}.2xV3^m.T.R/p17/24MB.

*19)   Ad-E^m$\Delta$CAT^{300}.2xV3^m.T/G.PI*

   Peptide mapping and Elispot data indicate that specific regions of the

10   Gag protein may play significant roles in eliciting CTL response in animals

   immunized with the adenoviral vectors of the present invention.  To facilitate

   efficient expression of p17MA and p24CA by the adenoviral vector, DNA

   sequence encoding the protease (PI, DNA SEQ ID NO: 56, **Figure 55A**;

   amino acid SEQ ID NO: 57, **Figure 55B**) from the *pol* region of HIV strain

15   BH10 was inserted into a region downstream from the sequence encoding

   Gag in a shuttle vector pRAd-ORF6-G.PI (**Figure 35**).  As illustrated in **Figure**

   **35**, Gag and PI are expressed separately from a CMV promoter via a

   retroviral splicing donor (SD) and acceptor (SA) mechanism at two splicing

   acceptor sites, SA$_1$ and SA$_2$/ SA$_3$.

20   Both pLAd-E^m$\Delta$CAT^{300}.2xV3^m.T (**Figure 33**) and pRAd-ORF6/G.PI

   (**Figure 35**) were linearized using appropriate restriction enzymes and ligated

   to the backbone of the adenovirus (**Figure 4B**), resulting in the recombinant

   adenoviral vector Ad-E^m$\Delta$CAT^{300}.2xV3^m.T/G.PI.

25   *20)   Ad-E^m$\Delta$CAT^{300}.2xV3^m.T/G-PI*

   Alternatively, the HIV protease PI was expressed as a fusion protein

   with Gag by inserting a C residue at position nt1410 to allow *pol* to be read

   within the same reading frame of *gag*.  DNA [SEQ ID NO: 58] and amino acid

   [SEQ ID NO: 59] sequences of the Gag-PI fusion protein are shown in

30   **Figures 56A** and **56B**, respectively.  As illustrated in **Figure 36**, Gag and PI

   are expressed from the same CMV promoter within the same reading frame.

   The resulting shuttle vector is designated as pRAd-ORF6/G-PI.

Both pLAd-E$^m$ΔCΔT$^{300}$.2xV3$^m$.T (**Figure 33**) and pRAd-ORF6/G-PI

(**Figure 36**) were linearized using appropriate restriction enzymes and ligated

to the backbone of the adenovirus (**Figure 4B**), resulting in the recombinant

adenoviral vector Ad-E$^m$ΔCΔT$^{300}$.2xV3$^m$.T/G-PI.

B.　　Immune responses of animals to the adenoviral vaccine against HIV
antigens

Experimental mice were inoculated with the adoviral vaccine

constructed above, Ad.tat.env.IL2 (also designated as "Ad-E.T.R/IL2" as

described above, section A, subsection 1)), to elicit immune response to the

HIV antigens expressed by this vector. Immunogenicity of the adenoviral

vector was determined by measuring titers of antibody against HIV tat and

env.

**Figures 6** and **7** show the immunogenicity of Ad.tat.env.IL2 against the

HIV Env protein in two groups of mice, respectively. These groups of

C57BL/6 mice (supplied by Charles River Laboratories. Wilmington, MA) were

injected intramuscularly with 10$^7$ pfu Ad.tat.env.IL2 on different dates as

indicated in the figures. Blood (about 150-500 µl for each animal) was

collected from four animals every two weeks following inoculation and serum

was prepared. At 77 days post-inoculation, these mice were re-challenged

with an additional 10$^7$ pfu of Ad.tat.env.IL2. Blood was collected from three

animals every day following secondary challenge. Titers of antibody elicited

against HIV tat and env were determined by ELISA against Ad.tat.env.IL2-

infected HeLa cell lysates.

Briefly, lysates of the HeLa cells infected with Ad.tat.env.IL2 were

prepared as follows. HeLa cells were infected with Ad.tat.env.IL2 at a

multiplicity of infection (MOI) of 20. Fourty-eight hours post infection, HeLa

cells were harvested and resuspended in a buffer that contained 1% TritonX-

100. A post-nuclear supernatant was obtained by centrifuging the lysates at

15,000 x g for 5 min. The lysates were diluted to 10 µg/ml for coating wells of

ELISA plates. Standard ELISA assays were performed to measure OD450 of

the sera and relative titers of antibody against HIV tat and env proteins were

calculated by normalizing against the mean of the CD450 of mouse pre-immunization sera.

As shown in **Figure 6**, the three mice in this group had strong immune responses to the HIV antigens expressed by the adenoviral vector

5    Ad.tat.env.IL2, with the highest titer of antibody against HIV antigens reached in about 42 days post inoculation. The second inoculation with Ad.tat.env.IL2 boosted the immune reponse again and very high titers were achieved within about 5 days of the second inoculation.

As shown in **Figure 7**, the three mice in this group also had strong

10   immune responses to the HIV antigens expressed by the adenoviral vector Ad.tat.env.IL2, with the highest titer of antibody against HIV antigens reached in about 70 days post inoculation. The second inoculation with Ad.tat.env.IL2 boosted the immune reponse again and very high titers were achieved within about 5 days of the second inoculation.

15   **Figures 12 A-B** show the antibody production elicited by the recombinant adenoviral vectors Ad.3C.env.gag (also designated as "Ad-3C/E$^m\Delta$C$\Delta$T$^{300}$-G" as described above, section A, subsection 2)) in mice. C57BL/6 mice were injected intramuscularly with 10$^7$ pfu Ad.3C.env.gag. At 77 days post-inoculation, these mice were re-challenged with an additional

20   10$^7$ pfu Ad.3C.env.gag. Relative antibody titers of these mice were determined by ELISA against purified recombinant Gag (obtained from the NIH AIDS Research and Reference Reagent Program, Bethesda, MD) at week 10 post-immunization (or prime) **(Figure 12A)** and week 14 post-prime/week 3 post-boost **(Figure 12B)**. As shown in **Figures 12A** and **12B**,

25   the mice inoculated with Ad.3C.env.gag had strong immune responses to the HIV antigen Gag.

**Figures 13 A-B** show the antibody production elicited by the recombinant adenoviral vectors Ad.3C.env.rev.gag (also designated as "Ad-3C/E$^m\Delta$C$\Delta$T$^{99}$.T.R-G" as described above in section A, subsection 3)) in mice.

30   C57BL/6 mice were injected intramuscularly with 10$^7$ pfu Ad.3C.env.rev.gag. At 77 days post-inoculation, these mice were re-challenged with an additional 10$^7$ pfu Ad.3C.env.rev.gag. Relative antibody titers of these mice were

determined by ELISA against recombinant purified Gag at week 10 post-immunization (or prime) (**Figure 13A**) and week 14 post-prime/week 3 post-boost (**Figure 13B**). As shown in **Figures 13A** and **13B**, the mice inoculated with Ad.3C.env.rev.gag had strong immune responses to the HIV antigen

5    Gag.


C.    Activation of cytotoxic T lymphocytes (CTL) by immunization with the adenoviral vaccines against HIV antigens


10    Activation of cytotoxic T lymphocytes (CTL) by immunization with the adenoviral vaccine against HIV antigens was measured by using two independent assays: an IFNγ assay and a granzyme A assay. The IFNγ and granzyme assays were designed to detect antigen-specific activation of T-cells. IFNγ is secreted by activated CTL and $T_H1$ helper T cells which function

15    specifically in the cellular immune pathway. Granzyme A is also secreted by activated CTL. The basic approach is to incubate splenocytes with target cells that express antigens of interest and look for secretion of IFNγ or granzyme A into the medium.


20    *1)    IFNγ assay*

This assay is a modification of the standard $^{51}$Cr-release lytic assay (Current Protocols in Immunology, Coligan et al., eds.) except that the target cells are not radiolabeled prior to incubation with the splenocytes. Detailed procedures for this assay are described in Di Fabio et al. (1994) "Quantitation

25    of human influenza virus-specific cytotoxic T lymphocytes: correlation of cytotoxicity and increased numbers of IFN-gamma-(or IFNγ-) producing CD8+ T cells" Int. Immunol. 6:11-9. Briefly, about 1x10$^5$ splenocytes were incubated with 10$^5$ target cells (e.g., infected with appropriate viruses carrying the target antigens) in a total volume of 100 µl. Cells were incubated for 4h at

30    37°C. IFNγ was measured by ELISA from 25 µl medium.

Activation of CTL in mice inoculated with the adenoviral vaccine against HIV antigens was determined by using the IFNγ assay described

above. Briefly, twelve C57BL/6 mice were injected intramuscularly with $10^7$ pfu Ad.tat.env.IL2. Spleens were harvested from 4 inoculated mice at the time points indicated in **Figures 8A-C**. Splenocytes were activated by incubation with B16-F1 cells (a melanoma cell line from C57BL/6, ATCC No:

5    CRL-6323) that had been infected with Ad.tat.env.IL2. At day seven after stimulation, activated splenocytes were mixed with B16-F1 cells infected with the indicated viruses. IFNγ secretion into the medium was determined by ELISA (R&D Systems, Minneapolis, MN).

     **Figures 8A-C** show percent increases in the amount of IFNγ secreted

10   into the medium over the period of time ranging from 4-8 weeks post inoculation. As shown in **Figure 8A**, secretion of IFNγ increased significantly in splenocytes of the four mice harvested 4 weeks post inoculation with Ad.tat.env.IL2. In contrast, little increase in IFNγ secretion occurred when the splenocytes were incubated with B16-F1 cells infected with an adenoviral

15   vector expressing non-specifc protein β-Gal (Ad.lacZ) or uninfected B16-F1 cells.

     Secrection of IFNγ increased more in the splenocytes of mice harvested 6 weeks post inoculation as shown in **Figure 8B**. Noticeably, there was near 100% increase in secrection of IFNγ in splenocytes of mouse 5

20   **(Figure 8B)**.

     Secrection of IFNγ increased more dramatically in the splenocytes of mice harvested 8 weeks post inoculation as shown in **Figure 8C**. There was more than 300% increase in secrection of IFNγ in splenocytes of mouse 11 **(Figure 8C)**.

25       These results demonstrate that strong humoral immune responses against HIV, such as induction of high titer antibody and activation of CTL specifically targeting HIV antigens, have been achieved by inoculating animals with the adenoviral vaccine expressing both HIV viral antigens and an immuno-stimulator such as IL-2. The immune responses resemble those

30   during a recovering of viral infection diseases. These results tend to show that the genetic vaccines of the present invention that mimics natural viral infection hold great promises as efficacious vaccines for humans against HIV.

## 2) Granzyme A assay

Granzyme A assay was performed using a protocol modified from the one described in Deitz et al. (2000) "MHC I-dependent antigen presentation is inhibited by poliovirus protein 3A" Proc. Natl. Acad. Sci. 97:13790–13795. The granzyme A assay described in Deitz et al. was a modification of a protocol described in: Kane et al. (1989) "Cytolytic T-lymphocyte response to isolated class I H-2 proteins and influenza peptides" Nature (London) 340:157–159.

Granzyme A Assays were performed following similar procedures as for IFNγ assays with the following exceptions. Granzyme A secretion into the medium was determined by an enzymatic assay. Units of granzyme A were determined by calculating the slope of activity during the linear phase of the reaction. One unit of granzyme A was defined as the amount of enzyme required to convert the substrate to 1 $OD_{405}$ in one hour.

Briefly, about $1 \times 10^5$ activated splenocytes and about $1 \times 10^5$ target cells were incubated together as for the IFNγ assays. Granzyme A activity was determined by combining 20 µl medium with 180 µl reaction mixture (0.2 mM BLT (N-α–benzyloxycarbonyl-L-lysinethiobenzyl ester, Sigma, St. Louis, MO), 0.22 mM DTNB (5,5'-dithio-bis(2-nitrobenzoicacid, Sigma, St. Louis, MO)) in 96-well plates and incubating at room temperature. Absorbance at 405 nm was monitored over a period of several hours. Slopes of enzyme activity were determined for the linear phase of the reaction and converted to units of enzyme.

**Figure 9** shows increases in the amount of granzyme A secreted into the medium for splenocytes of mice harvested 8 weeks post inoculation. As shown in **Figure 9**, secretion of granzyme A increased significantly in splenocytes of the four mice harvested 8 weeks post inoculation with Ad.tat.env.IL2. In contrast, much less granzyme A secretion occurred when the splenocytes were incubated with B16-F1 cells infected with an adenoviral vector expressing non-specifc protein β-Gal (Ad.lacZ), an adenoviral vector expressing both hepatitis B surface antigen and IL-2 (Ad.HBsAg/IL2) or

uninfected B16-F1 cells. Similarly, there is little spontaneous granzyme A secrection in these splenocytes not incubated with the target cells.

**Figure 14A** shows the results of the granzyme A assays for series 1 mice at various time points indicated, including week 4, 6, 8 post-

5      immunization and week 12/1, 13/2, 14/3 (prime/boost) post-secondary inoculation with Ad.3C.env.gag.

**Figure 14B** shows the results of the granzyme A assays for series 2 mice at various time points indicated, including week 2, 4, 6, 8 post-immunization with Ad.3C.env.gag.

10      These results, obtained by using the granzyme A assay independent from the IFNγ assay, again demonstrate that strong activation of CTL specifically targeting HIV antigens was induced by inoculating mice with the adenoviral vaccine expressing both HIV viral antigens and an immuno-stimulator such as IL-2. These results also support the belief that the genetic

15      vaccines provided by the present invention hold great promises as efficacious vaccines for humans against HIV.


*3)*      *ELISPOT Assay*

ELISPOT assays were performed to determine CTL activation in mice

20      inoculated with the recombinant adenoviral vectors, Ad.3C.env.gag and Ad.3C.env.gag.rev. C57BL/6 mice were inoculated with $10^7$ pfu Ad.3C.env.gag or Ad.3C.env.gag.rev. Mice were sacrificed at two-week intervals and splenocytes were prepared (see Current Protocols in Immunology, Coligan et al. eds.). At week 11, mice were inoculated with a

25      second dose of $10^7$ pfu of Ad.3C.env.gag or Ad.3C.env.gag.rev. $2 \times 10^5$ splenocytes were incubated with $4 \times 10^4$ MC57G cells (ATCC #CRL-2295) that had been infected with vaccinia viruses expressing either Env, Gag, or Rev, in 96-well, mouse IFNγ, ELISPOT plates (R&D Systems, Minneapolis, MN) for 30h. Non-specific activation was monitored following the addition of 4

30      μg/ml PHA (Sigma, St. Louis, MO) instead of antigen-expressing cells.

IFNγ spots were visualized as per the kit instructions and counted. Wild type and recombinant vaccinia viruses were obtained from the NIH AIDS Research and Reference Reagent Program, Bethesda, MD.

**Figures 15A** shows the ELISPOT results for the four mice in serie1 at

5   week 13/2 post-prime/boost with Ad.3C.env.gag. **Figures 15B** shows the ELISPOT results for the four mice in serie1 at week 13/2 post-prime/boost with Ad.3C.env.rev.gag. These results indicate that immunization of mice with the genetic vaccines of the present invention induced strong activation of CTL against HIV Gag.

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### 3.     Genetic Vaccine Against Hepatitis B Virus

Embodiments of the genetic vaccine against hepatitis B virus and methods of their construction are described in detail as follows.

15   1)     <u>Construction of replication-defective adenoviral vaccines against hepatitis B virus</u>

Two adenoviral vectors, Ad.HBsAg.IL2 and Ad.HBcAg.IL2, were constructed to carry the coding sequences for a hepatitis B surface antigen

20   (HBsAg) and a HBV core antigen (HBcAg), respectively. In the same vector, DNA sequence encoding interleukin-2 (IL-2) was also included and expressed by a promoter different from that for expressing the viral antigen. This design is believed to be able to ensure high level expression of both the viral antigens and the immuno-stimulator IL-2 and to enhance immunogenicity of

25   the adenoviral vaccine. As shown by experimental data presented in the next section, both of these two adenoviral vectors are capable of eliciting strong and long-lasting immune responses in animals against hepatitis B antigens.

These two adenoviral vectors, Ad.HBsAg.IL2 and Ad.HBcAg.IL2, were constructed using strategies similar to those for constructing the adenoviral

30   vaccines against Ebola virus as described in detail above.

a)     *Ad.HBsAg.IL2*

Briefly, full length HBsAg (with a silent mutation caused by deletion of Xba I site) was inserted into the left end (E1 region) of the adenoviral genome using a shuttle vector pLAd (**Figure 4A**, left side), resulting in a shuttle vector pLAd-CMV-HBsAg.

5    The sequence encoding IL-2 (with a silent mutation caused by deletion of Xba I site) was inserted into E4 region of the adenoviral genome using the shuttle vector pRAd (**Figure 4A**, right side), resulting in a shuttle vector pRAd-CMV-IL2.

Both pLAd-CMV-HBsAg and pRAd-CMV-IL2 were linearized using
10   appropriate restriction enzymes such as Xba I and EcoRI and ligated to the backbone of the adenovirus (**Figure 4B**), resulting in the recombinant adenoviral vector designated Ad.HBsAg.IL2.

a)    *Ad.HBcAg.IL2*

15   Briefly, sequences encoding full length HBsAg (with a silent mutation caused by deletion of Xba I site) and full length HBcAg were inserted into the left end (E1 region) of the adenoviral genome using a shuttle vector pLAd (**Figure 4A**, left side). HBsAg and HBcAg are expressed separately from another CMV promoter via a retroviral splicing donor (SD) and acceptor (SA)
20   mechanism at two splicing acceptor sites, $SA_1$ and $SA_2$. The shuttle vector produced is designated pLAd-CMV-SD/$SA_1$-HBsAg-$SA_2$-HbcAg.

Sequences encoding multiple immuno-stimulators, including IL-2 (with a silent mutation caused by deletion of Xba I site), INF-$\gamma$, and GMCSF, were inserted into E4 region of the adenoviral genome using the shuttle vector
25   pRAd (**Figure 4A**, right side). These three immuno-stimulators are expressed separately from another CMV promoter via a retroviral splicing donor (SD) and acceptor (SA) mechanism at three splicing acceptor sites, $SA_1$, $SA_2$, and $SA_3$. The shuttle vector produced is designated pRAd-CMV-SD/$SA_1$-IL2-$SA_2$-INF$\gamma$-$SA_3$-GMCSF.

30   Both pLAd-CMV-SD/$SA_1$-HBsAg-$SA_2$-HbcAg and pRAd-CMV-SD/$SA_1$-IL2-$SA_2$-INF$\gamma$-$SA_3$-GMCSF were linearized using appropriate restriction enzymes such as Xba I and EcoRI and ligated to the backbone of the

adenovirus (**Figure 4B**), resulting in the recombinant adenoviral vector designated Ad.HBcAg.IL2.

5     2)     <u>Immune responses of animals to the adenoviral vaccines against HBV antigens</u>

Experimental mice were inoculated with the adoviral vaccine constructed above, Ad.HBsAg.IL2 and Ad.HBcAg.IL2, to elicit immune response to the hepatitis B surface antigen and core antigen expressed by 10   these two vectors, respectively. Immunogenicity of these adenoviral vectors was determined by measuring titers of antibodies against HBsAg and HbcAg, respectively.

*a)*     *HBV surface antigen (HBsAg) antibody titers*

15      CD-1 mice (Charles River Laboratories, Wilmington, MA) were injected intramuscularly with several different concentrations of Ad.HBsAg.IL2: $10^0$, $1\times10^6$, $1\times10^7$, $1\times10^8$, $5 \times 10^5$, $5 \times 10^6$, and $5 \times 10^7$ pfu virus. **Figure 10A** shows the relative Anti-HBsAg antibody titers measured for sera harvested from mice inoculated with $1 \times 10^5$ and $5 \times 10^5$ pfu. Serum in each 20   measurement was diluted 1:500. **Figure 10B** shows the relative Anti-HbsAg antibody titers measured for sera harvested from mice inoculated with $1\times10^7$ and $1\times10^8$ pfu. Serum in each measurement was diluted 1:1500.

To measure the relative titers of the Anti-HbsAg antibody elicited by Ad.HBsAg.IL2, blood (about 150-500 µl) from each animal was collected from 25   immunized mice every two weeks and serum was prepared. Blood was incubated at room temperature for 2-3 h to allow for clotting. The blood was then chilled overnight at 4°C to shrink the clot. Unclotted liquid was transferred to a clean tube and centrifuged at 2000 x g for 5 min. The supernatant was transferred to another clean tube. Sodium azide ($NaN_3$) was 30   added to 0.05% as a preservative. Small aliquots were kept at 4°C for short-term storage. Long-term storage was at -80°C.

Relative anti-HBsAg titers were determined by ELISA against recombinant HBsAg purified from yeast (from Aldevron, LLC, Fargo, ND). As shown in **Figure 10A**, the mice in group 1 had increasingly strong immune responses to HBsAg expressed by the adenoviral vector, Ad.HBsAg.IL2,

5   within 8 weeks post inoculation. This vector with a titer as low as $5 \times 10^5$ pfu was sufficient to elicit high levels of antibody specifically against HBsAg.

**Figure 10B** shows the immunogenicity of Ad.HBsAg.IL2 with higher titers. As shown in **Figure 10B**, immunogenicity of Ad.HBsAg.IL2 increased dramatically as the titer of the adenoviral vector was increased from $1 \times 10^7$

10   pfu to $1 \times 10^8$ pfu.

These results demonstrate that the adenoviral vector expressing both hepatitis B surface antigen and IL-2 can induce strong immune response specifically targeting the viral antigen in mice inoculated with this vector. These results also support the belief that the genetic vaccines provided by the

15   present invention hold great promises as efficacious vaccines for humans against hepatitis B virus.

b)   *HBV core antigen (HBcAg) antibody titers*

Groups of C57BL/6 mice (Charles River Laboratories, Wilmington, MA)

20   were injected intramuscularly with $1 \times 10^7$ pfu Ad.HBcAg.IL2 on different dates. Blood was collected from four animals every two weeks following inoculation and serum was prepared. At 91 days (Group 3, **Figure 11A**) or 84 days (Group 4, **Figure 11B**) post-inoculation, mice were re-challenged with an additional $1 \times 10^7$ pfu virus. Blood was collected from three animals every day

25   following secondary challenge. Antibody titer was determined by ELISA against recombinant HBcAg purified from E. coli (from Chemicon International, Inc., Temecula, CA).

As shown in **Figure 11A**, mice in group 3 had strong immune response to the hepatitis core antigen HBcAg expressed by the adenoviral vector

30   Ad.HBcAg.IL2, with the highest titer of antibody against HBcAg reached in about 28 days post inoculation. The second inoculation with Ad.HBcAg.IL2

boosted the immune reponse again and very high titers were achieved within about 3 days of the second inoculation.

As shown in **Figure 11B**, mice in group 4 also had strong immune response to the hepatitis core antigen HBcAg expressed by the adenoviral

5    vector Ad.HBcAg.IL2, with the high titer of antibody against HBcAg reached in about 34 days post inoculation. The second inoculation with Ad.HBcAg.IL2 boosted the immune reponse again and very high titers were achieved within about 3 days of the second inoculation.

These results demonstrate that the adenoviral vector expressing both

10   hepatitis B core antigen and IL-2 can also induce strong immune response specifically targeting the viral antigen in mice inoculated with this vector. These results once again support the belief that the genetic vaccines provided by the present invention hold great promises as efficacious vaccines for humans against hepatitis B virus.